

Modulating the Hedgehog pathway

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Martin Strand

2. Abbreviations

General marks: *Species taxonomy names and gene names and are written in cursive*. Although there are exceptions, Human genes/proteins are generally written in capital letters (PTCH), while mouse (Ptc) and *Drosophila* (Ptc) genes/proteins are written with a capital letter only in the first letter of the name. Unless a species is specified or a part of the context, the mouse protein/gene name is used.

A	Anterior	G-protein	Heterotrimeric GTP-binding
AmphiHh	Amphioxus Hh protein		regulatory proteins
Arrb2	β -arrestin 2	GRK2	G-protein coupled receptor
BCC	Basal cell carcinoma		kinase 2
BMP	Bone morphogen protein	GSK3	Glycogen synthase kinase 3
Boc	Brother of Cdo	Hedge	N-terminal Hh fragment
Botv	Brother of Tout velu		(signaling component)
Cdo	Cell adhesion molecule- related/down-regulated by oncogenes	Hh	Hedgehog
		Hh-N	N-terminal Hh fragment
			(signaling component)
Ci	Cubitus interruptus	Hh-Np	Dual lipid modified Hh-N
CiA	155 kDa transcriptional activator form of Ci		fragment (p for processed)
		HPLC	High performance (pressure)
CiR	75 kDa transcriptional repressor form of Ci		liquid chromatography
CKI	Casein kinase I	HIP	Hedgehog interacting protein
Cos2	Costal2	Hog	C-terminal (autocatalytic)
D	Dorsal		domain of Hh
Dhh/DHH	Desert hedgehog	HSC	Hedgehog signaling complex
Disp/DISP	Dispatched	HSPG	Heparin sulfate proteoglycan
Dlp	Dally-like protein	Ihh	Indian hedgehog
Dpp	Decapentaplegic	Ihog	Interference hedgehog
EXT1/2	Exostosin 1/2	IP	Intra peritoneal
En	Engrailed	LC	Liquid chromatography
F	Floorplate	Lpp	Lipophorin
Fgf10	Fibroblast growth factor 10	LRP2	Low density lipoprotein-related protein 2
Fu	Fused, a serine/threonine kinase	LRX	Liver X receptor
Gas1	Growth arrest-specific gene 1	MEF	Mouse embryonic fibroblast
Gli1-3/GLI1-3	Glioma-associated oncogenes 1- 3	MN	Motor neurons
		MP	Micro particles
GliA	Transcriptional activator form of Gli2/3	MS	Mass spectrometry
		N	Notochord
GliR	Transcriptional repressor form of Gli2/3	NMR	Nuclear magnetic resonance spectroscopy
Glis	Gli-similar	NPC1	Niemann–Pick type C1 disease
Glypican	Glycosylphosphatidylinositol linked HSPG		gene/protein
		NO	Nitric oxide
GPCR	G-protein coupled receptor	OHC	hydroxycholesterol/oxysterol
GPI	Glycosylphosphatidylinositol	OSBP	Oxysterol binding protein

ORP	OSBP related protein	Sotv	Sister of Tout velu
P	Posterior	SSD	Sterol sensing domain
PI4P	Phosphatidylinositol-4 phosphate	STT4 kinase	Staurosporine and temperature sensitive PI4P kinase
PKA	Protein kinase A	SuFu	Supressor of Fused
PKC	Protein kinase C	TGF β	Transforming growth factor β
Ptc/PTCH	Patched	TLC	Thin layer chromatography
Ptc-1-3	Patched gene homologs 1-3	TIC	Total ion current
Ptr	Patched related gene/protein	Ttv	Tout velu
RND	Resistance nodulation division	V	Ventral
SANT	Smo antagonist	V0-V3	Interneurons
SIM	Selected ion monitoring	Wg/Wnt	Wingless
Shh/SHH	Sonic hedgehog	ZPA	Zone of polarizing activity
Ski/Skn/SKI	Skinny hedgehog		
Smo	Smoothened		

3. Publications included

Paper I

Wilson SR, **Strand MF**, Krapp A, Rise F, Herstad G, Malterud KE, Krauss S. Hedgehog antagonists cyclopamine and dihydroveratramine can be mistaken for each other in *Veratrum album*. J. Pharm. Biomed. Anal. 2010. 53(3):497-502.

Paper II

Wilson SR, **Strand MF**, Krapp A, Rise F, Petersen D, Krauss S. Hedgehog antagonist cyclopamine isomerizes to less potent forms when acidified. J. Pharm. Biomed. Anal. 2010. 52(5):707-13.

Paper III

Strand MF, Wilson SR, Dembinski JL, Holsworth DD, Khvat A, Okun I, Petersen D, Krauss S. A novel synthetic smoothened antagonist transiently inhibits pancreatic adenocarcinoma xenografts in a mouse model. PLoS One. 2011;6(6):e19904. Epub 2011 Jun 15.

Paper IV

Larsen HR, **Strand MF**, Grimsø A, Olsen PA, Dembinski JL, Rise F, Lundanes E, Greibrokk T, Krauss S, Wilson SR. High sensitivity measurements of active oxysterols with automated filtration/filter backflush-solid phase extraction-liquid chromatography-mass spectrometry. J Chromatogr A. 2012 Feb 28. [Epub ahead of print]

4. Introduction

Inter- and intracellular signaling is a key biological feature of all organisms, from single cell organisms like bacteria to multi-cellular organisms like vertebrates. Cellular signaling occurs in several ways; by direct contact between cells (juxtacrine signaling), by secreted ligands that act on the ligand producing cell itself (autocrine signaling), by secreted ligands that act on neighboring cells over short distances (paracrine signaling) and by secreted ligands that act over large distances (endocrine signaling). The ligands can be peptides, lipids and other molecules, and each ligand can activate a specific response by targeting a certain receptor (or receptors) on the surface or inside the receiving cell. Signal pathway activation is often followed by a signaling cascade where the signal is amplified by the use of kinases, phosphatases and secondary messengers. The activation of a signaling cascade can have multiple outcomes, including alterations in the intracellular concentration of specific ions, such as Ca^{2+} , changes in enzymatic activity, and regulation of transcription factor activity. These changes alone or in combination control cell function and behavior. Cellular signaling plays a vital role in biological processes like development, tissue homeostasis, tissue repair, cell growth and immunity. Changes or errors in cell signaling are linked to various human diseases and disorders, and the identification of molecules that can activate (agonize) or inhibit (antagonize) signaling pathways is an important field of medical research and drug discovery.

4.1 History of the Hedgehog pathway

The Hedgehog (Hh) pathway is a highly conserved signaling pathway that plays a vital role in embryonic development, as well as being involved in several human disorders and diseases. The first member of the Hedgehog pathway was identified in 1980 [2] by Christiane Nüsslein-Volhard and Eric Wieschaus who performed a series of genetic screens on the fruit fly *Drosophila melanogaster* in the late 1970s and early 1980s. The fruit flies have several strengths as a genetic model organism. The small flies are easy to maintain in large numbers due to their small size, simple needs and quick generation time. The genetic screens were based on chemically induced random mutations that were linked to various phenotypes in the easily observable developmental stages of the fruit fly, especially the larval body segmentation. Based on this technique, the two researchers identified more than 50 genes that were involved in embryonic development, and thus laid the basis for much of our current understanding of development in the fruit fly as well as vertebrates. Their work was recognized in 1995

when they earned the Nobel Prize in Physiology or Medicine together with the developmental geneticist Edward B. Lewis. One of the mutations they induced resulted in a distinct phenotype of short larva where the ventral cuticular denticles formed a dense lawn instead of the distinct segmented bands in the wild type (Figure 1).

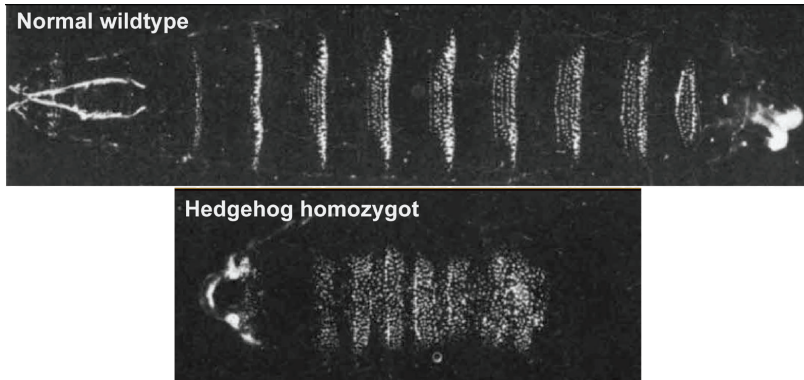


Figure 1. Ventral cuticular pattern of fixed *Drosophila* larva shortly after hatching, showing normal wild type larva, and a larva homozygous for hedgehog. In the Hedgehog mutant the segmentation is lost, with the resulting cuticle lawn phenotype appearing (modified from (Nüsslein-Volhard and Wieschaus 1980) [2]).

Due to the close resemblance between this stubby and “hairy” phenotype and a hedgehog, the gene harboring the loss-of-function mutation was named *Hedgehog* (*Hh*). Early research suggested that the *Hh* gene encoded a protein involved in cell-cell signaling [28,29], and this was later confirmed when the *Drosophila Hh* gene was cloned [30–33]. It was quickly apparent that the *Hh* gene was highly conserved in vertebrates, as homologues was identified in *Mus musculus* (mouse) [34], *Danio rerio* (zebrafish) [35], *Gallus gallus* (chicken) [36], *Xenopus laevis* (frog) [37], *Rattus rattus* (rat) [38], *Homo sapiens* (human) [39,40] and many other species. In total three distinct *Hh* homologues were identified in vertebrates, contrasting the one *Hh* gene found in *Drosophila*. While two of the vertebrate homologues were named after hedgehog species, *Indian Hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*), the third member was named *Sonic Hedgehog* (*Shh*) after the Sega videogame character Sonic the Hedgehog [34]. The gene *Patched* (*Ptc*) was identified alongside hedgehog in the *Drosophila* segmentation studies [2]. *Ptc* was later identified as a target of *Hh* in *Drosophila* segmentation, and Ingham et al. suggested early that *Ptc* could be the *Hh* receptor [29]. Still the focus shifted for a while to another gene implied in *Hh* signaling, called *Smoothened* (*Smo*), that was proposed as the *Hh* receptor [41,42]. Further studies, however, revealed that *Ptc* was indeed the receptor for the *Hh* protein ligands [43–46]. The transcription factor *Cubitus interruptus* (*Ci*) was identified

as the key response element in Hh signaling [47,48], and later three mammalian Ci-related proteins with similar functions, named Glioma-associated oncogens 1-3 (Gli1-3), were found [49,50].

4.2 The Hedgehog pathway in development

Cellular signaling is a key aspect in the developmental systems that drive the development of a fertilized egg (a zygote) into an adult organism. Important developmental systems are differentiation, growth, pattern formation, segmentation and morphogenesis. The zygote is totipotent, which entails that it has the potency to develop into every kind of cell that the adult organism contains, as well as extraembryonic tissues such as the placenta. Each cell type maintains a particular pattern of gene expression, and the development of a specific cell type is called differentiation. Gene expression is regulated by transcription factors and signaling proteins and a certain pattern of gene expression in a differentiated cell can be maintained or altered depending on the signals the cell receives. Embryogenesis in placental mammals start with the cleavage of the zygote into eight totipotent cells, and these cells form a spherical shape called a morula. The outer cells of the morula then develop into trophoblast cells that give rise to the extraembryonic tissues, while the cells inside the morula develop into the inner cell mass. The inner cell mass contains pluripotent cells that can give rise to the three germ layers, the endoderm, mesoderm and ectoderm. These cells are no longer defined as totipotent as they cannot form extraembryonic tissues. The growth of the embryo is achieved by increasing the number of cells through repeated cellular divisions, called cell proliferation, and as the embryo grows the cells differentiate into specific cell lineages and further into specific cell types. While the cells of the inner cell mass are pluripotent, they lose a part of their potency as they develop into specific cell lineages. In the adult body, cells are classified as adult stem cells, multipotent progenitor cells, unipotent precursor cells and differentiated cells. Multipotent cells have the potential to give rise to a limited number of cellular lineages, while unipotent cells only have the capacity to develop into one cell type.

Pattern formation is the process by which equivalent cells develop more complex structures and organizations of cell fate, and is for instance important in determining which cells develop into which organs. Pattern formation is controlled by genes, and is best understood in the anterior-posterior axis formation in *Drosophila* larva. Pattern formation involves signaling between adjacent cells and gradients of secreted signaling ligands, called morphogens. Segmentation is the division of a body plan into a series of

repetitive segments, an example being the human spinal cord, while morphogenesis refers to the process by which a part of a developing system forms a defined shape.

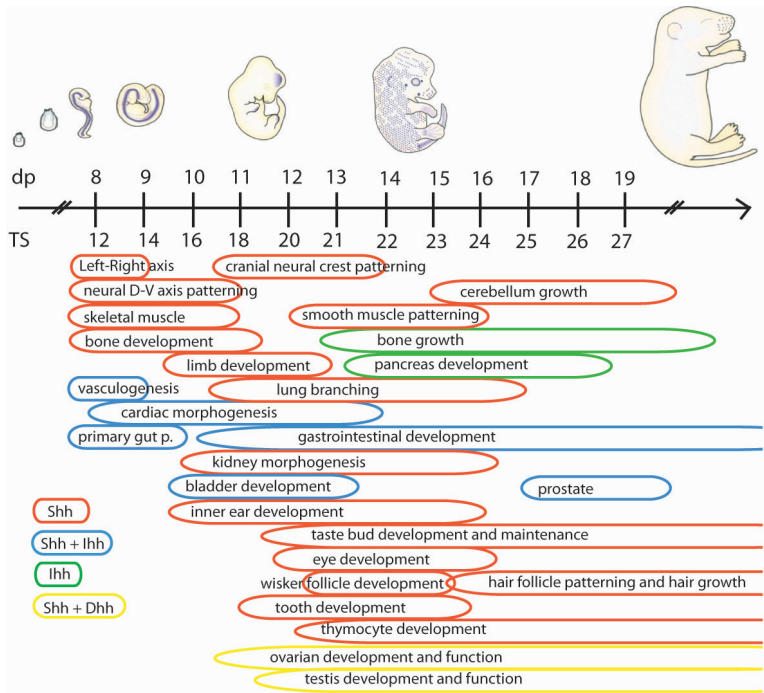


Figure 2. Hh signaling in mouse development and tissue patterning, including a cartoon of *Ptch* expression during mouse embryonic development, as well as bars depicting the approximate embryonic stages when Shh, Ihh and Dhh are involved in development and patterning (modified from Varjosalo and Taipale, 2008 [3]). For references see [3].

The first insights into the role of the Hh protein in development were its involvement in the segmentation of the fruit fly larva [2], and in the development of adult fly appendages [51–55]. Hh and another signal that was also identified in early *Drosophila* studies, *Wingless (Wg)* [56], establishes the parasegmental boundary between the separate segments in the fruit fly larva epidermis. While Wg is expressed by a narrow band of cells on the posterior (P) side of the segment, Hh is expressed by a narrow band of cells on the anterior (A) side, and the Wg and Hh expressing cells lie in juxtaposition on each side of the parasegmental boundary. Hh expression is maintained by Wg induced expression of *Engrailed (En)* and Wg expression is in turn regulated by Hh. The two ligands mutually thus exclude each others expression; maintaining a tight boundary.

An important feature of Hh signaling is that the ligands are able to both act on short distance, and act over a long range, controlling cell fates as a function of Hh concentration. In *Drosophila*, Hh is required for the development of the wings and legs as well as the patterning of the embryonic segments [57]. One such role is the formation of posterior/anterior axis and dorsal/ventral axis in the early embryo. In the development of the wing imaginal disc, the posterior cells express and secrete Hh proteins that act on cells in the neighboring anterior compartment to induce the expression of target genes including *Decapentaplegic* (*Dpp*). *Dpp* encodes a member of the transcription growth factor β (TGF β)/bone morphogen protein (BMP) family [51,54] that diffuses bidirectionally into both the A and P compartments, functioning as a long range morphogen that controls the growth and patterning of the cells throughout the wing [58,59] (Figure 3A). While Hh exerts an effect in the whole imaginal disk through its induction of *Dpp* expression, it also acts as a short range signal that regulates the expression of *Ptc* and *Engrailed* (*En*) within spatially limited regions [15].

Mammalian Hh homologues have critical roles in developmental stages as well as having important functions in the adult body. *Shh* has emerged as the most important of the three homologues, and is the one that has been most extensively studied. During mammalian development, *Shh* is involved in the patterning of several parts of the embryo, including the neural tube and midline structures in the brain, the limbs, the teeth, internal organs, the gut, and bone growth. The role of the Hh signaling in development has been reviewed in detail by Tabin et al. [60]. Figure 2 depicts the role of *Shh*, *Ihh* and *Dhh* in mouse tissue patterning and development, as reviewed by Varjosalo and Taipale [3]. In the patterning of the neural tube, cells in the notochord (N) and floor plate (FP) express *Shh* (Figure 3B). The *Shh* gradient that results from this expression is involved in the “programming” of different cell fates in the ventricular zone of the ventral neural tube [21,61–63]. Factors that influence the cellular response to a Hh signal include the threshold and the signal strength and duration. The spinal cord patterning by *Shh* involves the formation of several distinct neuronal subtypes (Figure 3B); the motor neurons (MN) and the interneurons (V0-V3). These subpopulations are defined by the strength and duration of the *Shh* signal. A similar mechanism is involved in the patterning of the digits in the developing limb, where a *Shh* gradient is involved in the patterning of the anterior-posterior axis. *Shh* is expressed in the posterior compartment of the limb bud that is called the Zone of Polarizing Activity (ZPA) (Figure 3C).

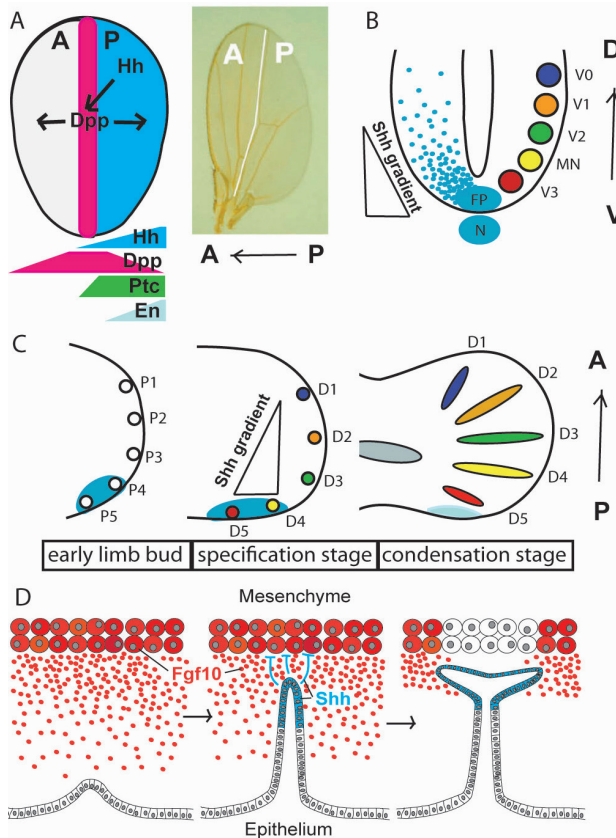


Figure 3. Hh signaling in development: A. Hh protein (blue) is produced by cells in the posterior (P) compartment of *Drosophila* wing imaginal disk, and diffuses towards the anterior (A) compartment where it induces neighboring A compartment cells to produce Dpp. Dpp (dark pink) then diffuses into both A and P compartments, controlling cell growth and patterning in the whole wing. Hh also acts as a local morphogen to specify patterning by regulating target gene expression, such as Ptc and En, within different spatial limits). (modified from Jia and Jiang, 2006 [7] and Strigini and Cohen, 1997 [15]). B. Shh protein produced by the notochord (N) and the floor plate (FP) in the ventral (V) part of the neural tube forms a gradient towards the dorsal side (D) that mediates the patterning of the neural tube, and the formation of motor neurons (MN) and interneurons (V0-V3) (modified from Jacob and Briscoe, 2003 [21]). C. In the patterning of the mouse limb Shh protein (blue) forms a gradient from the P compartment towards the A compartment, directing the patterning of the presumptive region of digits (P1-5) and the resulting digit morphological identities (D1-5) through the different developmental stages (modified from Tamura et al. 2011 [25]). D. Lung branching is mediated by the combined effect of Fgf10 and Shh signaling between the mesenchyme and epithelium. Mesenchymal cells secrete Fgf10 (red) that induce proliferation and growth of epithelial cells. Fgf10 also induces expression of Shh (blue) in the epithelial cells (left), which in turn inhibits Fgf10 expression in neighboring mesenchymal cells (middle). The continued Fgf10 expression further away on each side of the bud continues to induce growth, leading to a branching of the growth towards the two sides (right) (modified from Robb W. Glenny, 2011 [27]).

Interestingly researchers have found that if they take out a part of the tissue from the ZPA and transplant it from the posterior side to the anterior side of the limb bud, the digit development is patterned from both sides of the limb bud, resulting in a mirror image duplication of the digits [64,65]. These examples of anterior/posterior and dorsal/ventral axis formation all build on the same principle, namely the generation of a signaling gradient by Hh ligands that act as a morphogen. Shh is also required for the correct formation of the lungs [66–68] where it is involved in lung branching morphogenesis [27,69]. Lung branching is mediated through interaction between the epithelium and the distal mesenchyme. The mesenchyme secretes Fibroblast growth factor-10 (Fgf10), which in turn activates epithelial proliferation and growth, as well as expression and secretion of Shh. As the budding epithelium grows towards the mesenchyme, secreted Shh inhibits expression of Fgf10 in the neighboring mesenchyme. Further away in the mesenchyme the Fgf10 expression is retained, resulting in a branched growth of the epithelial bud towards the Fgf10 expressing regions on each side (Figure 3D).

While playing a vital role in vertebrate development, Shh signaling is also important in the adult body. There are reports of the involvement of Shh in the maintenance of stem cells in various tissues [70], including neural [70–76], mammary [77], gastric [78], and hematopoietic stem cells [79,80], but as the term stem cells in some cases is more loosely defined, this may be more progenitor like cell populations. Shh has also been reported to be critical for regeneration of the pulmonary epithelium [81], prostate epithelium [82] and the exocrine pancreas [83]. Many of these tissues are the site for development of cancers that harbor Hh signaling, as will be detailed below. Shh is required for the proper formation of the hair follicle bulb and hair production during the postnatal hair cycle [84]. Hh signaling may have a role in regulating insulin in the murine pancreas [85], and Shh has been identified as an important factor in wound healing [86–89], possibly by promoting angiogenesis in the wounded tissue [87].

4.3 The Hedgehog signaling pathway

In brief, the key signaling components in the hedgehog pathway are the ligands, Hh and Shh, the cell membrane receptors, Ptc and Smo, lipophilic signal mediators and the signaling complex containing the transcription factors Ci/Gli1-3. In absence of Hh, Ptc acts by suppressing the function of Smo by regulating the distribution of cholesterol and oxysterols in the cells and by regulating the synthesis of the phospholipid phosphatidylinositol-4-phosphate (PI4P). In the lack of active Smo, the Ci/Gli signaling complex releases the repressor form of the Ci/Gli proteins. The repressor form of the

transcription factors is then translocated to the nucleus where it blocks the transcription of target genes. When Hh is present, it binds and blocks the function of Ptc, thus releasing Smo from Ptc mediated suppression. Activation of Smo then leads to a change in the activity of the Ci/Gli signaling complex, resulting in the release of activating Ci/Gli proteins. The activating form of the transcription factors then translocate to the nucleus to activate the transcription of target genes. While active Ptc and Smo are located in the cell membrane in *Drosophila*, they are localized in the primary cilium in vertebrates. The Hh pathway's double-negative regulation, with the Hh ligand inhibiting Ptc and preventing it from inhibiting Smo, resulting in gene expression, is quite different from the "normal" scheme of cellular signaling in which a ligand activates a receptor and a signal cascade. The important role played by cholesterol, oxysterols and PI4P in mediating the Hh signal is also a unique feature of this pathway. The signaling mechanisms and protein interactions involved in the Hh signaling pathway are further detailed below.

4.3.1 Maturation and movement of hedgehog ligands

As briefly described above, the Hh proteins act as cell-cell signaling molecules. The *Drosophila* Hh and its mammalian counterparts are all synthesized as a pre-protein that enters the secretory pathway. This pre-protein contains three parts, a signal sequence, a C-terminal domain that has an autocatalytic activity, and an N-terminal domain that contains the signaling properties. After a removal of the signal sequence, the Hh precursor is further matured as the C-terminal domain catalyzes the cleavage of the unfinished precursor into two fragments [90–92], the N-terminal Hh fragment (Hh-N) (also named "hedge") and the C-terminal fragment (named "hog"). The C-terminal domain also catalyzes the addition of a cholesterol moiety to the C-terminal part of Hh-N [93,94]. The Hh-N fragment is further modified by the addition of a palmitate to a cysteine residue near the N-terminal end of Hh-N by an acyltransferase called Skinny Hedgehog (Ski) (murine Skn, human SKI or HHAT) [95–97], forming a dual lipid modified Hh ligand (Hh-Np) (p for processed). Hh-Np is then transported to the cell membrane where the hydrophobic property of the cholesterol and palmitate moieties tethers it to the phospholipid bilayer. While membrane tethered Hh-Np can act on neighboring cells, the Hh gradients displayed in the developmental stages still require a soluble form of Hh. It seems illogical that a lipid-modified protein should be able to diffuse over a long range, as lipid modifications normally anchor proteins to the membrane. Paradoxically, although the non-lipid modified Hh-N diffuses much further than Hh-Np, it can only activate low level targets, probably due to a low affinity for the plasma membrane and for proteins that are implicated in Hh movement [98]. Hh-Np has

been shown to form multimeric complexes where the hydrophobic moieties cluster at the core of the complex, and these multimeric complexes are capable of long range signaling [95,99,100]. Active multimeric Hh complexes can be isolated from tissue-culture-cell supernatants, and vary in size between 158 and 4.000 kDa, equivalent to 6-160 times the monomer size [101]. The release of Hh-Np is dependent on the activity of a Ptc related protein called Dispatched (Disp) [102–105]. Hh-Np co-localizes with the Apo-lipoprotein Lipophorin (Lpp) in lipoprotein particles in *Drosophila*, and lipoproteins may be required for Hh signaling in the fly [106,107]. It has also been demonstrated that Shh can be carried by micro particles (MP), small fragments of cell membrane that is shed from the plasma membrane of activated/apoptotic T-cells [108]. Similarly small membrane fragments, called nodal vesicular parcels have been implied in left right patterning in mouse development [109]. MP's containing Shh may also contribute to reparative neovascularization after ischemic injury, by regulating the nitric oxide (NO) pathway and genes involved in angiogenesis [110]. In *Drosophila* the movement of modified Hh protein across several cells requires the expression of glycosaminoglycan transferases like Tout velu (Ttv), Sister of Tout velu (Sotv) and Brother of Tout velu (Botv), enzymes that are involved in the synthesis of heparin sulphate proteoglycans (HSPG), implicating that HSPG's are required for the movement of Hh ligands [111,112]. The exotosin (EXT1 and EXT2) proteins are homologues of Ttv and Sotv that have similar functions in mammals [113,114]. The glycosylphosphatidylinositol (GPI) linked HSPG (Glypicans) Dally and Dally-like protein (Dlp) are substrates of Ttv that facilitate movement of Hh ligands along cell membranes [115–117] by binding of Hh to the heparin sulfate moieties. Dally and Dlp also recruit Lpp to the membrane of the Hh secreting cells, mediating the interaction between Lpp and the Hh ligand [106]. It has been suggested that the mechanism of Hh release is that complexes of Hh, Lpp and glypicans are released from the cells by cleavage of the GPI anchor of Dally/Dlp [118]. In addition, metalloproteases may be involved in the release of Hh from cells [119]. Other membrane-anchored proteins that influence Hh transport and gradients have been described, and Hh gradient formation has been reviewed in detail by Torroya et al [120].

Several proteins have been identified that affect the reception of the Hh ligand in the receiving cells, including the transmembrane proteins Interference hedgehog (Ihog) and Brother of Ihog (Boi) in *Drosophila*, and the vertebrate homologs “Cell adhesion molecule-related/down-regulated by oncogenes” (Cdo) and Brother of Cdo (Boc). These proteins facilitate binding of Hh ligands to the Ptc receptor in a synergistic manner [121,122], and their expression is down regulated in response to Hh signaling in mammals. The membrane protein Growth Arrest-Specific Gene 1 (Gas1) has an activity similar to Cdo/Boc, promoting Hh binding to Ptc [123,124]. Similar to Ihog/Boi and

Cdo/Boc, Gas1 is down regulated in response to Hh, serving as a negative feedback to Hh signaling.

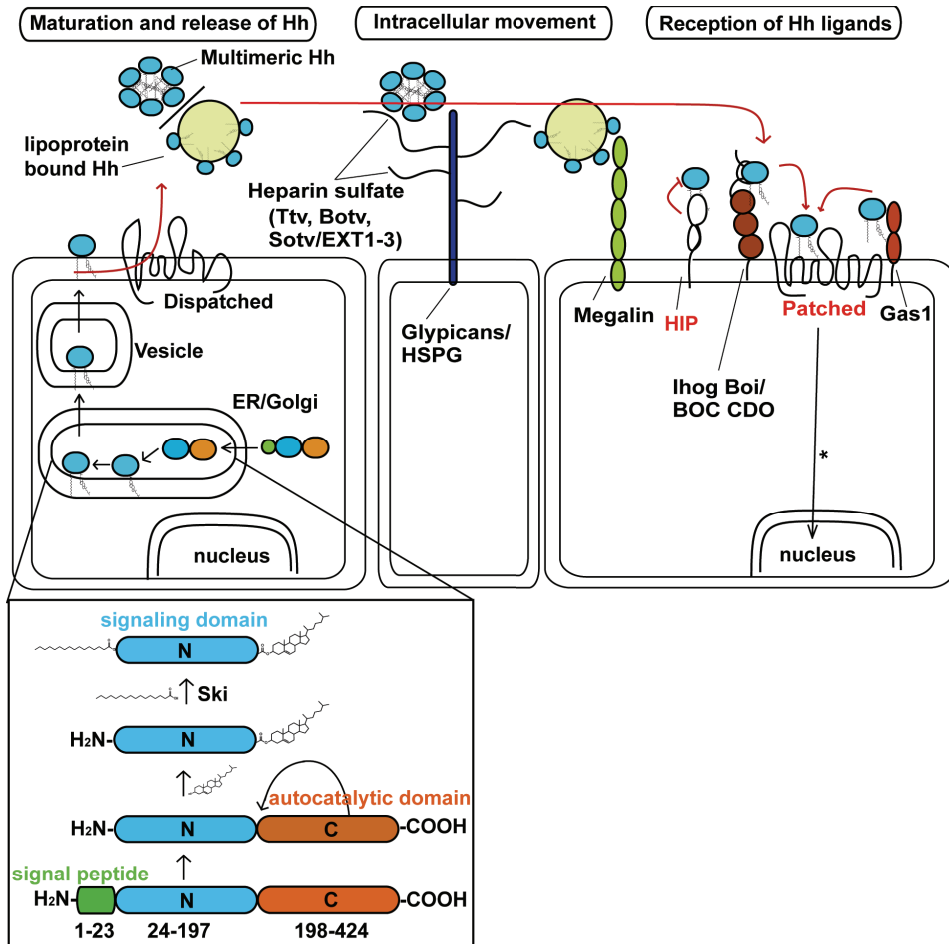


Figure 4. Maturation, release, movement and signal reception of the Hh ligands. The maturation of the Hh protein involves signal sequence cleavage, autocatalytic cleavage and addition of cholesterol and subsequently, addition of palmitic acid. Mature Hh proteins associate with the cell membrane and are packaged into multimeric units (and lipoproteins) by Dispatched, facilitating their release. Movement of Hh across cells requires HSPG and Glypicans, and the synthesis of the heparan sulfate side chains on HSPG and Glypicans require the activity of Ttv, Botv, Sotv/EXT1-3. Megalin, Ihog/Boi/BOC/CDO and Gas1 positively influence Hh recruitment and binding to Ptc, while HIP and Ptc sequesters Hh ligands. Upon binding Ptc, the Hh signal pathway is activated. Parts of the figure are modified from Varjosalo and Taipale, 2008 [3], Jiang and Hui, 2008 [12], Wilson and Chuang, 2010 [9].

Megalin, also known as Low density lipoprotein-related protein 2 (LRP2) is a multi-ligand binding receptor in the plasma membrane of endothelial cells, that binds lipoproteins [125,126] and is involved in the uptake of a range of different molecules and proteins into cells [127]. Megalin is involved in the recovery of vitamin D in the renal tubule [128], and has been shown to bind the Hh protein as a positive regulator of the pathway [129]. Mice that lack Megalin experience defects in forebrain development, with a holoprosencephalic phenotype similar to Shh knockout mice [130]. Hh proteins that bind to Ptc is internalized and degraded, and thus the Ptc protein does not only mediate the signal reception, but also acts to sequester Hh ligands [131]. As Hh induces Ptc expression, Ptc acts in a negative feedback loop from Hh pathway activation to limit the range of diffusing Hh. Similarly, a protein called hedgehog-interacting protein (HIP) also binds and sequesters Hh ligands, limiting the range and strength of Hh signals. HIP is expressed both as a cell membrane bound and as a soluble extracellular protein [131–133]. For clarity Hh-Np will be referred to as Hh hereafter. The maturation, secretion, movement and reception of Hh ligands are detailed in Figure 4.

4.3.2 Hh signal reception; Patched, Smoothed, lipids and cilia.

There are two vertebrate homologues of Ptc, Ptch1 and Ptch2, and while Ptch1 function seems to be equivalent to Ptc, the function of Ptch2 function is not well known. Still there are indications that Ptch2 may have overlapping functions to Ptch1 in certain tissues [134–136]. Ptch is a 12-pass multimeric transmembrane domain protein [29,43,44,46,57,137–140], that have a sterol-sensing domain (SSD), and that share sequence similarity to resistance nodulation division (RND) family of proton-driven transmembrane transporter proteins, often called RND permeases [141]. Proteins carrying SSD domains are generally involved in sterol metabolism or vesicle transport [142], while RND permeases are membrane pumps that have rather broad substrate specificities. It seems that, similar to RND permeases, Ptch forms trimers in the cell membrane [143]. In addition to the protein Disp that is needed for Shh release, the closest relative of Ptch is a protein encoded by the Niemann–Pick type C1 disease gene (*NPC1*). NPC1 was discovered through its association with Niemann–Pick syndrome, a disease in cholesterol storage/metabolism [144,145]. Smo is a 7-pass transmembrane domain protein that is similar in structure to a family of 7-transmembrane heterotrimeric GTP-binding regulatory proteins (G-protein)-coupled receptors (GPCR). Although some reports suggest that Smo could function like other GPCR's by activating G α i-family members [146–151] this still remains to be proven [152].

While it was first believed that Ptc blocks Smo by direct binding, this model was later discarded in favor of a catalytic model, as it was discovered that Ptc can inhibit Smo at a stoichiometry of 1:50 [153]. Several lines of evidence now point towards Ptc being a sterol pump, and towards Smo being a sterol receptor [153]. Interestingly the data does not point towards one specific ligand, and the activity of Smo can be modulated by a range of molecules, from endogenous metabolites such as cholesterol [154], oxysterols [155,156] and provitamin D3 and/or vitamin D3 [157] as well as a number of synthetic small molecules that antagonize and agonize Smo activity [14,158]. Ptc has also been implied in the transport of some of these molecules. For instance, there is some evidence that Ptc acts as a pump that increases cholesterol efflux from the cell [154]. In support of this model, the binding of Shh to Ptc led to an increase in the intracellular levels of cholesterol. The addition of cholesterol also induced Hh pathway activity similarly to Shh and the Hh agonist purmorphamine [154]. Oxysterols, or hydroxycholesterols (OHC), are a group of oxygenated 27-carbon cholesterol derivatives that emerge from direct sterol oxidation or from metabolic pathways involved in the formation of sterols, steroids and bile acids. Several oxysterols have been implied in Hh signaling as factors that lead to ciliary translocation and activation of Smo [155,156], and recent data indicate that oxysterols can act as allosteric modulators of Smo [159]. An identified connection between mutations in enzymes involved in cholesterol synthesis and phenotypes associated with mutations in the Hh pathway was first interpreted as evidence that cholesterol modification of the Hh protein was essential. However, cholesterol synthesis was later shown to be essential for correct transmission of the Hh signal in the receiving cell at the level of Smo [160], supporting the idea that cholesterol and oxysterols are activators of Smo. Mutation based analysis of the Ptc protein has also shown that a functional SSD domain is essential for Smo repression [161]. It has also been reported that another sterol related molecule, pro-vitamin D3 (7-dehydrocholesterol), can be pumped out of cultured cells by Ptc, and that provitamin D3 and its metabolite, vitamin D3, are potent antagonists of Smo [157]. In addition it has been proposed that Ptc regulates Smo in *Drosophila* by recruiting lipoproteins and a lipoprotein derived lipophilic Smo antagonist to the endosomes that Smo is trafficking through [162]. Ptc has also been implied in the regulation of the intracellular levels of the phospholipid phosphatidylinositol-4 phosphate (PI4P), possibly through inhibition of the STT4 kinase (staurosporine and temperature sensitive PI4P kinase) [163], the kinase that forms PI4P. Smo activation was also shown to require PI4P [163].

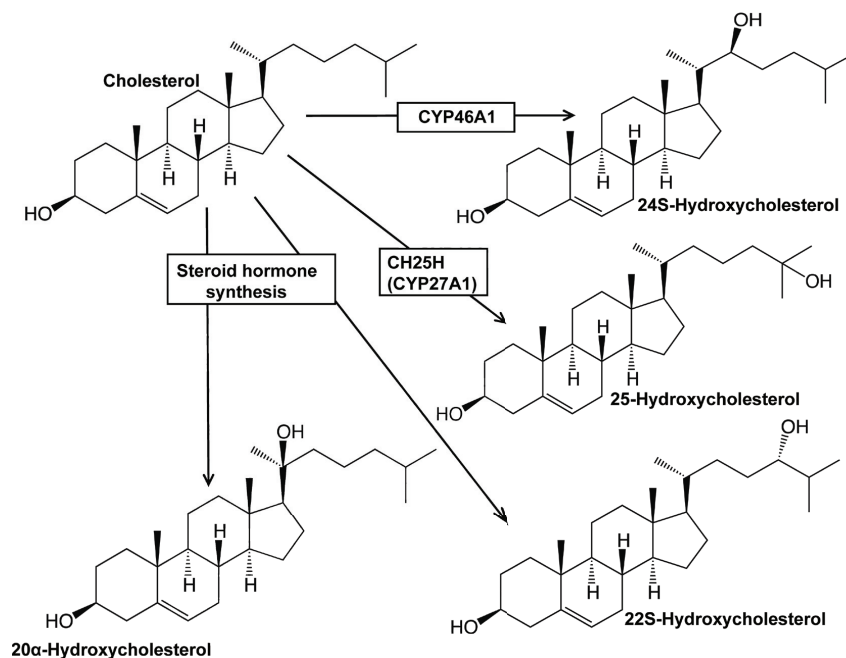


Figure 5. Structure of cholesterol and Hh pathway activating hydroxycholesterol (OHC) products, 24S-OHC, 25-OHC, 22S-OHC and 20α-OHC. The enzymes or pathways involved in synthesis of the oxysterols are also indicated. The figure is modified from Olkkonen et al, 2012 [1].

While implied in Hh signaling, oxysterols are also involved several biological mechanisms, including cholesterol homeostasis [164], apoptosis [165–167], atherosclerotic plaque formation [168], cell differentiation [169] and signaling [170]. Several oxysterols occur naturally, including 25-OHC (cholest-5-ene-3β, 25-diol), 24S-OHC (cholest-5-ene-3β, 24-diol), and 27-OHC (cholest-5-ene-3β, 27-diol) [171]. Oxysterols have been described to exert their effect in the cells by acting as ligands for the nuclear receptors of the liver X receptor (LXR) family [172–174]. However oxysterols appear to have many other effects in cells in addition, and an oxysterol binding protein (OSBP) [175,176] and several OSBP related proteins (ORP's) have been identified. The OSBP/ORP family has been characterized both in mouse [177] and human [178], and the human family consists of 12 OSBP/ORP family members, encoding at least 16 different transcripts, many of which appear in short and long splice variants [179]. The OSBP family is involved in the control of lipid metabolism, sterol transport, signal transduction, and vesicular transport [170,180–187] but have not yet been linked to the Hh pathway. The oxysterols found to activate Hh signaling are 20α-OHC, 22S-OHC, 24S-OHC and 25-OHC [155,156], and while 24S-OHC is synthesized by cholesterol-24-

hydroxylase (encoded by *CYP46A1*) in the CNS, and transferred into the circulation, 25-OHC is either a product of cholesterol autooxidation or created by enzymatic action of cholesterol-25-hydroxylase (CH25H) [188] or CYP27A1 [189]. 22R-OHC and 20R-OHC are formed in steroidogenic tissues as intermediates in steroid hormone synthesis [190]. Hh related oxysterols and their enzymatic synthesis are depicted in Figure 5.

When trying to understand Ptc and Smo function it is important to take their cellular localization into account. In *Drosophila*, these two proteins cycle between internal endosomal compartments and the cell membrane [191] and their localization is regulated by the Hh ligand. In absence of Hh, Ptc is primarily localized in the plasma membrane where it acts by inhibiting the accumulation of Smo [137,192], by inducing conditions that favor the inward movement of Smo [193]. The binding of Hh protein to Ptc leads to an internalization of the Hh-Ptc complex through a dynamin-dependant endocytosis [194], and the complex is further targeted for degradation in lysosomes [195]. Being released from suppression by Ptc, Smo is then activated and translocated to the cell membrane. Smo has been shown to exist in both active and inactive forms, and is thought to be activated in a two step process [196–198]. In *Drosophila*, Smo activation involves PKA and CK1 mediated hyperphosphorylation of the cytoplasmic tail of Smo [199,200] and dimerization of Smo proteins. This dimerization is required for translocation to the membrane [201], and Smo mutants lacking the phosphorylation sites are defective in Hh signaling [199]. In contrast, vertebrate Smo lacks PKA and CK21 phosphorylation sites [201], and several reports indicate that the kinase phosphorylating Smo in vertebrates is the GPCR kinase 2 (GRK2) [202–204].

Hh signaling in vertebrates diverge from *Drosophila* in several ways, the most important being the requirement for the primary cilia in vertebrate Hh signaling [205–210]. The primary cilium is a structure found in most eukaryotic cells that is about 3 μ M long. It consists of a basal body, one of the centrioles in the cell, and a microtubule shaft, called an axoneme, that is enclosed by the cell membrane, sticking out from the cell like an antenna. The basal body and the daughter centriole is part of a larger structure that is called the pericentriolar material or the centrosome. Mutations in intraflagellar transport (IFT) proteins involved in anterograde ciliary transport have been reported, resulting in phenotypes similar to Hh loss of function phenotypes [209,211,212]. The role of cilia seems to be specific for vertebrates. Hh signaling is impaired with the loss of the primary cilium in mammals and zebrafish, but not in *Drosophila*. The protein Iguana, which was originally implicated in the regulation of the Hh pathway in zebrafish, has been shown to have a role in ciliogenesis [213], and the loss of Iguana results in a lack of ciliary pit formation and axonemal outgrowth [214]. In the absence of Hh ligands in vertebrates, Ptc is found in the membrane, where it is enriched in the shaft and around the base of

the primary cilium [215]. Ptch inhibits the phosphorylation and activation of Smo, thus preventing Smo from being translocated to the primary cilium where it can further activate the pathway [205,210,215–217]. Similarly to *Drosophila*, vertebrate Ptch is internalized when binding Hh ligands [215] and further processed for degradation [192]. Upon the release from Ptch suppression, Smo is phosphorylated, changes its conformation [201] and translocates from the endosomes and into the primary cilium [198]. Activated Smo associates with B-arrestin 2 (Arrb2), leading to endocytosis via clathrin coated pits [202], and Arrb2 may regulate Smo translocation to the cilia [218]. The translocation of Smo to the cilia is not enough to activate the pathway, supporting the idea that the Smo protein can exist in several forms, possibly in an inactive, a partially active and a fully active form [196,198].

The differences in the Hh pathway between vertebrates and the invertebrate *Drosophila* lead to several questions about the evolutionary origin of this pathway. By evaluating the function and presence of different parts of the Hh signaling pathway across species, more light has been shed on its evolutionary origin. The Hh protein appears to have evolved from two separate proteins, the N-terminal “Hedge” domain and the C-terminal “Hog” domain [219,220]. While evolutionary relatives of both Hedge and Hog have been identified in poriferans [219,220], cnidarians contain both a complete Hh gene and separate Hedge and Hog genes, indicating that the merge of these two genes into one Hh gene happened after the last common ancestor of these two phyla [219]. Amphioxus, which is a close relative to vertebrates contain a single Hh gene, called AmphiHh, indicating that the duplication of the Hh gene is specific to the vertebrate evolutionary tree [221]. AmphiHh is expressed in the notochord and ventral neural tube of amphioxus, similar to the expression of Shh and Shh-like proteins in vertebrates. According to one model, the Hh pathway evolved from a system that was controlling lipid homeostasis. In this system Smo is thought to have acted as a sensor of a lipid transported by Ptch, possibly cholesterol or a sterol derivative of cholesterol. Increased lipid levels could then have induced Smo mediated expression of Ptch, resulting in an increased efflux of this lipid from the cell [222]. The evolutionary fusion of the Hedge and Hog domains would further have provided the means for coupling of cholesterol to the Hedge protein, catalyzed by Hog, resulting in a Hh protein capable of blocking Ptch function. The earliest evolutionary relatives of Ptch are the RND permeases, which secrete hopanoids out of bacteria. Hopanoids are a sterol like class of molecules that are byproducts of bacterial metabolism. The nematode *Caenorhabditis elegans* lack homologues of Hh and Smo, but has three Ptch gene homologues, *ptc-1/3* [223]. In addition as many as 24 *ptc-related* (*ptr*) genes are present. The lack of Smo indicates that the nematode Ptch homologues may have retained a more ancestral role reminiscent of its evolutionary

origin. The absence of *ptc-3* in the nematode results in a late embryonic lethality due to an apparent defect in osmoregulation [223].

The immotile primary cilia in eukaryotic cells were for a long time overlooked and believed to be a nonfunctional cellular equivalent of vestigial organs, evolutionary relics from unicellular flagellate ancestors that have lost the function that was retained in motile cilia. But while only a few cell types in the body have motile cilia, most eukaryotic cells have a primary cilium. The cilium has most likely developed as an extension of the eukaryotic cytoskeleton organized by the centrioles, and is now recognized as an important sensory organelle that communicates signals into the cell based on both mechanical and chemical stimuli in the environment. While the primary cilia are required for the hedgehog pathway in developmental processes [224], they also play an essential part in sensory cells in the adult body. Odorant receptors in the nose and fluid flow mechanosensors in the kidney localize to cilia, while gigantic modified cilia make up the photoreceptors in the eye [225]. The coupling of Smo function to the primary cilium is interesting in light of the sensory role of the primary cilia and the role of GPCR's in mediating senses like taste, odor, vision and pain [226]. The characterization of the Hh pathway in planarians in comparison to the pathway in mammals and *Drosophila* has provided some insights into the merge of the Hh pathway and the cilium. In planarians, RNA interference mediated reduction of Cos2/Kif7, Fu or Iguana does not lead to measurable defects in the Hh pathway. In contrast, these proteins are essential for ciliogenesis in this organism [227]. These proteins may have been required for the assembly or function of motile cilia in an early eukaryotic ancestor. Further steps in the evolution of this pathway would then have been the association of Ptc, Smo and Gli with cilia, in addition to the loss of motility, resulting in immotile primary cilia with exclusively sensory capabilities. As a part of the ciliogenesis apparatus Cos2 and Fu may then have assumed roles in Hh signaling, and while the role of Fu have diminished in mammals, both Cos2 and Fu have been incorporated fully into the Hh pathway in *Drosophila*, with a following loss of primary cilia from fruit flies [228]. *Drosophila* Hh signaling is detailed in Figure 6, while vertebrate Hh signaling is detailed in Figure 7.

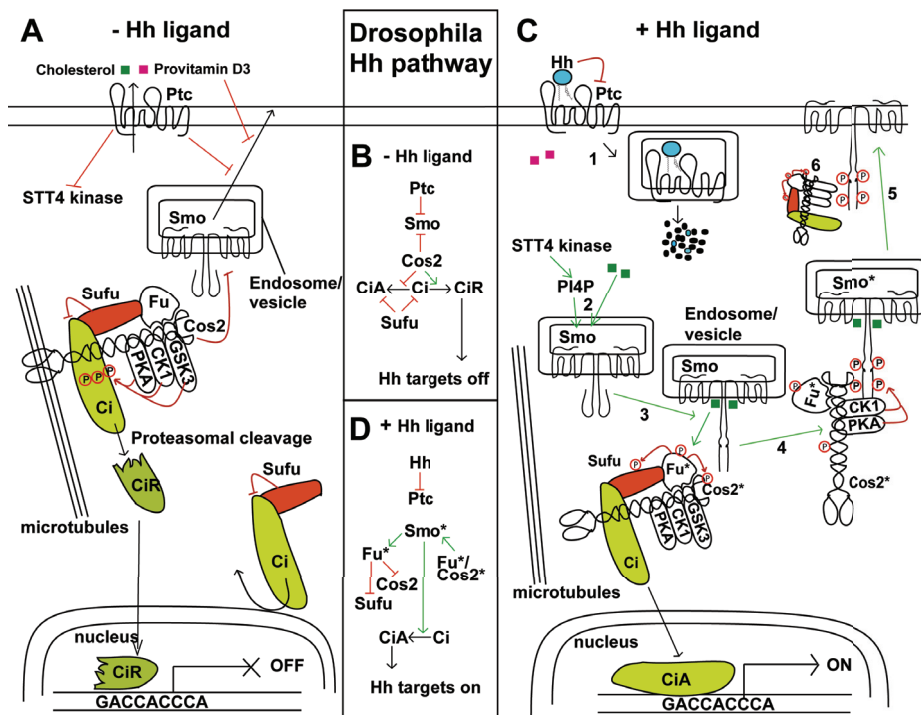


Figure 6. Hh signaling in *Drosophila*. A) In absence of Hh ligand, the Ptc receptor inhibits Smo from accumulating at the cell membrane by blocking STT4 kinase mediated PI4P synthesis, by removing cholesterol and possibly by exposing membrane associated Smo to provitamin D3. Ptc may also inhibit Smo while cycling through the same endosome compartments as Smo. Inactive Smo is also inhibited/retained by Cos2. Cos2 acts as a scaffold for the Hh signaling complex (HSC), bringing Ci, Fu and the kinases PKA, CK1 and GSK3 together. Upon phosphorylation, Ci is targeted for cleavage and partial degradation in the proteasomes, resulting in the release of CiR that represses Hh target genes. In addition Sufu inhibits Ci by retaining it in the cytosol, and Sufu is possibly also a part of the HSC. B) Schematic of the signaling events in the absence of Hh. C) 1. Upon binding of the Hh ligand Ptc is internalized and degraded. 2. In absence of Ptc, STT4 kinase produces PI4P, and in addition cholesterol (and/or oxysterols) is allowed to accumulate, resulting in Smo activation. 3. Activated Smo alters the HSC activity, leading to the release of the uncleaved activator form of Ci, CiA. This is likely mediated by phosphorylation and activation of Fu (Fu*), which in turn phosphorylates and inactivates Cos2 (Cos2*) and Sufu, leading to a dissociation of the HSC from the microtubules. 4. In addition Smo is further activated (Smo*) by hyperphosphorylation, possibly mediated by Fu*/Cos2* complexes. 5. Smo* then accumulates at the cell membrane. 6. There it continues to recruit and activate HSC for the release of CiA. CiA is transferred to the nucleus where it induces transcription of target genes. In presence of Hh some of the activity of Smo may be due to activation of G-protein (not shown). D) Schematic of signaling events in the presence of Hh. Parts of the figure are modified from Stegman et al. 2004 [16], Aikin et al. 2008 [19], Jiang and Hui, 2008 [12], Wilson and Chuang, 2010 [9], Li et al. 2011 [11] and Goetz and Anderson, 2010 [18].

4.3.3 The hedgehog signaling complex

In both *Drosophila* and vertebrates the signaling downstream of Smo centers around the function of a signaling complex called the Hedgehog signaling complex (HSC). In *Drosophila* the HSC contains the serine/threonine kinase Fused (Fu), the microtubule or membrane tethered kinesin-like protein Costal2 (Cos2), and the transcription factor Cubitus interruptus (Ci) [229]. In absence of Hh ligand Cos2 tethers the HSC to microtubules and endosomal membranes [16], while recruiting protein kinase A (PKA), protein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3). PKA, and subsequently GSK3 and CK1 then phosphorylate C-terminal domain of Ci, targeting Ci for cleavage and partial degradation into a transcriptional repressor (CiR) that translocates to the nucleus to suppress target genes [230–235]. Phosphorylated Ci is bound by a protein called Supernumerary limbs (Slimb) [236], that is a subunit of an ubiquitin ligase complex that targets phosphorylated proteins or peptides for ubiquitination and proteosomal degradation [237]. Slimb catalyses the ubiquitination of the carboxyl terminus of Ci, targeting it for degradation by the proteasome. In absence of active Smo, the translocation of full length Ci to the nucleus is also prevented by a protein called Suppressor of fused (Sufu) that binds and sequesters Ci in the cytoplasm [238,239]. In resting cells a large portion of Ci is bound by Sufu, while the rest is bound in the HSC [240]. Some of the HSC's in a cell may also exist as a non-microtubule-bound unit that interacts with Sufu [240,241], and binds to Smo [242]. In addition to its activity in the HSC, Cos2 is also thought to be involved in removing Smo from the plasma membrane, and sequestering Smo inside the cell [193].

Upon the release from Ptch suppression mediated by Hh proteins, two key events occur, one being the phosphorylation, activation and translocation of Smo to the membrane, and the other being a change in the processing of Ci in the HSC. Yet several of the proteins in the Hh pathway have more than one role, and the complex interactions make it difficult to establish a defined timeline of the signaling events. Activated Smo is primarily thought to induce a change in the HSC through its interaction with Cos2 and Fu, leading to phosphorylation of Fu. Fu in turn phosphorylates and alters the activity of Cos2 and Sufu, preventing these proteins from blocking the release and nuclear translocation of CiA [240,243]. In contrast, Fu and Cos2 have also been implied in the activation of Smo [244], indicating that Cos2 could mediate an interaction between Smo and the HSC in both its inactive and active state. The release of Smo from Ptch suppression may induce a change in the interaction between Smo and Cos2 resulting in a switch of the activity of PKA and CK1 from Ci to Smo [19,242], resulting in

phosphorylation and further activation of Smo, while the uncleaved activating form of Ci (CiA) is released [240,245–247].

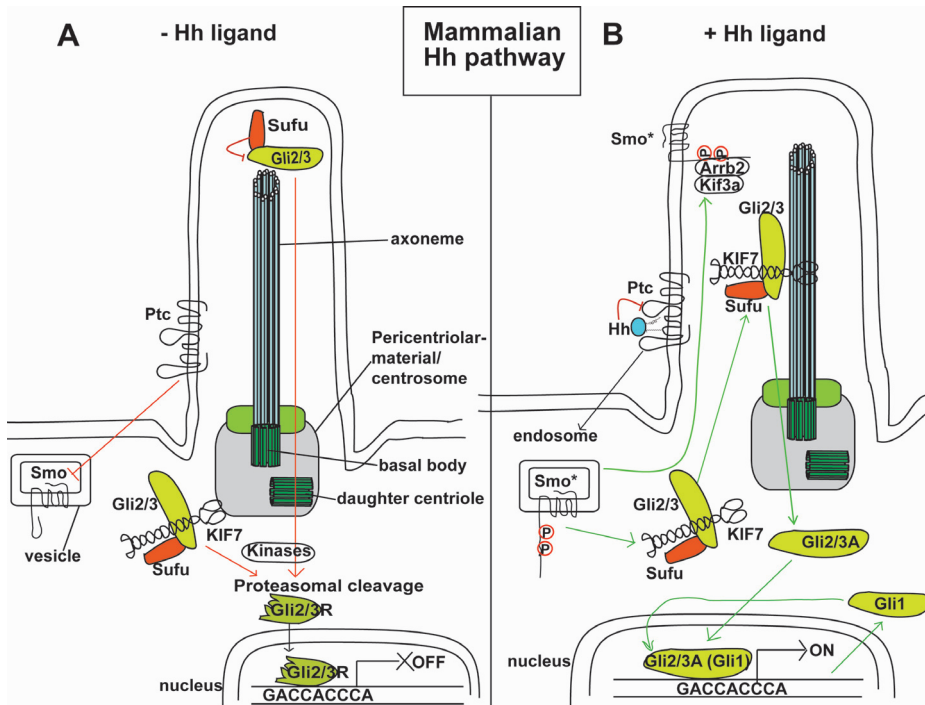


Figure 7. Hh signaling in vertebrates. A) In absence of Hh ligand, the Ptc receptor inhibits Smo from accumulating in the cilia, possibly by removing a sterol like lipid from the cell or from the ciliary environment. Ptc may also affect Smo while cycling through endosomes/vesicles containing Smo. In absence of active Smo, Gli is prevented from reaching the nucleus by Sufu, and Gli associates with a HSC possibly contain Kif7. Kif7 may recruit HSC's to the base of the primary cilia, but in the absence of active Smo, the HSC releases GliR that represses target genes. The amount of HSC's in the cilia in absence of Hh is low. B) In the presence of Hh, Ptc function is inhibited and Ptc is removed from the cilium by internalization into endosomes. This results in activation and translocation of Smo to the primary cilium, possibly involving Arrb2 and Kif3a. The amount of HSC in the cilia increases, and in presence of active ciliary Smo the HSC releases GliA that activates transcription of target genes in the nucleus. In presence of Hh and activated Smo, the suppressor function of Sufu is also inhibited, and Sufu is ubiquitinated and degraded, resulting in a lowered ability of the cell to retain Gli proteins in the cytosol. The involvement of cholesterol and other lipids like PI4P is not shown in this figure. Parts of the figure are modified from Wilson and Chuang, 2010 [9], Li et al. 2011 [11], Goetz and Anderson, 2010 [18], Scales and de Sauvage, 2009 [22] and Rubin and de Sauvage, 2006 [24].

There are also reports that Fu phosphorylation of Cos2 attenuates Cos2-Smo interaction and induces degradation of Cos2, releasing Smo from the suppression by Cos2 [244].

Cos2 has been shown to have kinsein like motor function, being able to move along microtubules [248], but it remains to be shown whether Cos2 does mediate movement of Smo or the HSC along microtubule, or whether Cos2 only tethers the complexes to the microtubule.

Table 1. Hedgehog pathway components in *Drosophila* and vertebrates (mouse)

<i>Drosophila</i> gene	Vertebrate (mouse)	Function
<i>Hedgehog (Hh)</i>	Shh, Ihh, Dhh	Secreted signaling ligand activating the Hh pathway
<i>Skinny hedgehog (Ski)</i>	Hhat (Skn)	Acyltransferase involved in Hh ligand palmitoylation
<i>Dispatched (Disp)</i>	Disp1, Disp2	Transmembrane protein involved in Hh ligand release
<i>Smoothed (Smo)</i>	Smo	Positive transmembrane transducer
<i>Patched (Ptc)</i>	Ptch1, Ptch2	Inhibits Smo translocation and activity, and possibly a sterol pump (cholesterol, provitamin D3). Also blocks STT4 kinase activity.
<i>Interference hedgehog (Ihog), Brother Ihog (Boi)</i>	Cdo, Boc	Ptch co-receptors
<i>Costal-2 (Cos-2)</i>	Kif 7, (Kif family)	Scaffold for Ci/Gli processing, positive and negative roles
<i>Fused (Fu)</i>	Fu (Stk36)	Positive transducer required for Sufu and Cos2 phosphorylation
<i>Suppressor of fused (Sufu)</i>	Sufu	Negative regulator of Ci/Gli proteins
<i>Cubitus interruptus (Ci)</i>	Gli1, Gli2, Gli3	Transcriptional activator and repressor of Hh target genes
<i>Dally, Dally-like protein (Dlp)</i>	GPC4, GPC6	Heparan-sulfate proteoglycan glypican involved in Hh movement and reception
<i>Tout-velu (Ttv), Sister of Ttv (Sotv), Brother of Ttv (Botv)</i>	Ext1-3	HSPG glycosylation (polymerization), involved in Hh movement
<i>Shifted (Shf)</i>	Wif	Secreted protein involved in Hh movement
<i>Hedgehog-interacting protein (HIP1)</i>	HIP	Negative regulator of Hh movement
<i>Protein kinase A (PKA)</i>	PKA	Ci/Gli and Smo phosphorylation, positive and negative regulator
<i>Casein kinase I (Ckl)</i>	CK1	Ci and Smo phosphorylation
<i>Shaggy (Sgg)</i>	GSK3 β	Ci and Smo phosphorylation
<i>Supernumerary limbs (Slimb)</i>	β -TRCP	F-box protein substrate recognition subunit of ubiquitin E3 ligase

The table is modified from Jia and Jiang 2006 [7].

There are three vertebrate homologues of Ci, namely Gli1, Gli2 and Gli3. The three homologues seem to have adopted different roles that, when combined, overlap with the regulatory properties of Ci. Gli2 and Gli3 are the primary mediators of Hh signaling [49,50,249,250]. Similarly to Ci, Gli3 and to a lesser extent Gli2 [251] undergo proteasome-mediated cleavage of the carboxyl-end to repressor forms (GliR) in the absence of Hh ligands. The primary role of Gli3 is to act as an Hh regulated repressor of transcription in absence of Hh, while the primary role of Gli2 is to act as an activator of transcription in the presence of Hh [21,252]. However, Gli2 can act as a repressor and Gli3 can act as an activator in some developmental contexts [49,251,253–255]. The third member, Gli1 acts solely as an activator [49,50], and is an important transcriptional target

of activated Gli2. Gli1 repressor function has never been observed [50,256,257]. In absence of Hh signaling Gli3 is cleaved and represses transcription of target genes while Gli2 is degraded and thus unable to activate transcription, resulting in a lack of Gli1 transcription [251]. Upon Hh pathway activation Gli3 is no longer cleaved and is thus unable to repress its targets, while Gli2 is translocated to the nucleus as an activator, leading to Gli1 transcription. Gli1 acts in a positive feedback loop, activating transcription of target genes, including itself. In support of the idea that the primary cilium is required for Hh signaling, not only Ptch and Smo, but Gli1, Gli2, Gli3 and Sufu are also trafficked through this organelle [196–198,212,215,216,258]. Like Ci in *Drosophila*, vertebrate Gli's are also found in a HSC. In absence of Hh and active Smo, the Gli proteins are retained in the cytoplasm as a part of the HSC's. In vertebrates this complex contains Fu, PKA, CK1, GSK3 β and possibly the Cos2 analog Kif7 [259,260]. Gli2-3 is phosphorylated by PKA in four sites [251,261], which targets Gli2-3 for further phosphorylations by CK1 and GSK3 β in a manner similar to Ci [232,234]. The hyperphosphorylated Gli proteins are then recognized by the Slimb homologue β -TrCP [251,262] before ubiquitination and degradation is mediated, and the truncated N-terminal repressor form is released. While the Slimb/ β -TrCP mediated ubiquitination of Ci/Gli2/3 leads to partial degradation, a E3 ubiquitin ligase system Hib/SPOP mediates ubiquitination that leads to complete Ci/Gli1 degradation [263,264]. Further a HECT family E3 ligase, Numb, promotes Itch-dependent ubiquitination and degradation of Gli1 [265]. With the requirement for cilia in Hh vertebrate Hh signaling, it is interesting to note that proteasomes localize to the base of the primary cilium. While Gli proteins that are captured by the HSC are processed to GliR in the absence of Hh, the nuclear localization of unprocessed Gli proteins is prevented by Sufu [266–268]. In addition Sufu acts as a nuclear co-repressor that removes Gli from the nucleus [269–271]. Sufu may also interact with Arrb2, Kif3a and IFT88 [216], but the cilium does not appear to be required for the Sufu mediated inhibition of Gli function [258,272]. Thus, the role of Sufu may be to prevent newly formed Gli proteins from entering the nucleus, allowing Gli to be retained in the cytosol where it can bind HSC's and be processed to a repressor. Thus Gli has to be processed through the cilia in order for Smo to inhibit Sufu [273], in order for Gli proteins to reach the nucleus. Gli protein activation is also prevented by other proteins; PKA can retain Gli1 proteins in the cytoplasm by phosphorylation of a PKA site in the nuclear localization signal peptide of Gli1 [274]. Upon binding of Hh to Ptch and the subsequent translocation of activated Smo to the cilia, HSC's containing Gli2 and Gli3 accumulate in the cilia. The presence of Smo leads to changes in the processing of Gli proteins in the HSC. The Gli suppressor Sufu, for instance, is ubiquitinated upon the activation of Hh signaling [266] which initiates its proteasomal degradation [275] leading to the release of the activating forms of Gli2/3 (GliA). GliA proteins translocate to the

nucleus to regulate transcription of downstream target genes including Gli1 and Ptch. The closely graded boundaries in early development also entail that the Hh pathway is not a on or off system, but that it can translate different Hh ligand concentrations and signal durations into various response levels.

While the hedgehog pathway in vertebrates and fruit flies has diverged in the requirement for the primary cilium, there are also other important differences. The phosphorylation sites for PKA, CKI and GSK3 are conserved between Ci and Gli1-3 [231,234] but in both mice and zebrafish the absence of PKA is enough to activate Hh signaling [276,277]. A recent kinase screen has identified Dyrk2 as a kinase that targets Gli2 for proteasomal degradation, while Dyrk2 in turn is modulated by MAP3K10 [278]. There are several Cos2 homologues in mammals in the family of Kif proteins, but a single Kif protein that functions like Cos2 has not been identified. While inactivation of Kif7 and Kif27 in cultured mammalian cells did not affect Hh signaling [279], *in vivo* studies still support that Kif7 is involved in the Hh pathway without interacting directly with Smo [259,260]. It is possible that several Kif's overlap in function as regulators of the transport of Smo and other signaling components in the cilia, both in retrograde and anterograde ciliary transport. The roles of Fu and Sufu have also diverged between fruit flies and vertebrates. While Fu can inhibit the degradation complex [280], as well as phosphorylating and inhibiting Sufu [270], Fu knock-out mice does not display phenotypes of impaired Hh signaling in embryonic development [281,282]. Thus Fu function may be fully dispensable in vertebrates, although Fu function is essential for fly development [283]. In contrast, while Sufu knock-out has very mild phenotypes in the fruit fly [284], Sufu knock-out mice have phenotypes that correspond to full pathway activation, equal to Ptch knockout mice [285].

Additional proteins that are not detailed here but that have been shown to affect Hh signaling are; Ren, a negative regulator preventing Gli translocation to the nucleus [286], Rab23, a Rab-GTPase vesicular transport protein that acts as a negative regulator of Hh signaling [287], Tectonic, a secreted glycoprotein that is required for both activation and inhibition of Hh signaling [288], Dyrk1, a kinase has been shown to enhance the transcriptional activity of Gli1 in the nucleus [289], MIM/BEG4, a actin-binding protein that modulates Hh signaling by regulating Gli-dependent transcriptional activation [290] and Sil, a protein that may be involved in the Hh pathway downstream of Ptc [291]. Two protein families of Krüppel-like zinc finger proteins that are related to Gli transcription factors have been identified called the Zic protein family [292] and the Gli-similar (Glis) protein family [293]. While Glis proteins regulate a number of physiological processes, and localize to the primary cilium, very little is known of how these transcription factors are regulated [293]. Members of the Zic protein family are

homologues of the *Drosophila* protein Odd-paired, and are involved in development [292], in some contexts in conjunction with Gli proteins. In addition, there are some indications that Zic and Gli proteins interact physically [294]. Key Hh pathway components are listed in Table 1.

4.3.4 Transcriptional targets of Hh signaling

The Hh signaling response is mediated by the binding of the Ci/Gli1-3 transcription factors to a Gli-consensus binding sequence, 'TGGGTGGTC' [295–298], in promoter and enhancer regions of target genes. The transcription factors act as both activators and repressors on the transcription of a number of genes that vary between organisms and tissues. There are several examples of graded responses to Hh signaling, often in conjunction with other signaling factors, including the establishment of the A/P boundary in wing disks and the tight segmental boundaries in *Drosophila* described above. In addition several response elements and enhancers, in addition to the Gli consensus sequence, may regulate the expression of each specific gene target. In *Drosophila*, Hh targets genes include *Dpp*, *Wg*, *Ptch*, *Col* and *En* [239,299]. Vertebrate targets include components of the pathway, *Ptch*, *Gli1* and *Hip*, as well as several proteins from various protein families including BMP [300] HOX [300], FGF [301], Myc [302], Cyclin [303], VEGF [304,305], Angiopoietin [305,306], and other proteins including PDGFR α [307], Bcl-2 [308,309], Bmi1 [310], Wnt [311], Hes1 [312], HNF-3b [298], SPOP [263]. GliR represses target genes when Hh is absent, and while moderate levels of Hh signaling leads to the de-depression these genes, higher levels leads to transcriptional activation. In mice, a total of 42 genes have two or more Gli consensus binding sequences in the enhancer regions [313]. There are also several Gli gene targets that act in feedback mechanisms on Hh pathway activity and the Hh protein. While Gli1 mediates an important positive feedback signal, the expression of *Ptch* and *Hip* reduce the movement of Hh ligands and retrains Hh signaling in a negative feedback loop. Factors involved in movement and reception of the Hh ligands, like *Ihog/Boi*, *Cdo/Boc* and *Gas1* are down regulated in response to Hh signaling, also functioning as negative feedback to Hh pathway activation.

4.4 Pathway interactions

Several of the Gli gene targets mentioned above are involved in other signaling pathways, including the secreted ligands BMP, FGF, VEGF and Wnt that can activate their corresponding signaling pathways in the Hh active or surrounding cells. Hh signaling, in turn, is regulated and modulated by a wide range of other pathways, and the

multiple pathway interactions make up a signaling network that controls cell fate and differentiation. The evolution of such pathway interactions was key to the development of multicellular organisms, in which single cells were required to cooperate with and respond to neighboring cells, while taking on different roles. Signaling pathways that affect Hh signaling can be divided into two categories. The first category is the pathways that induce expression of Hh ligands and the second, pathways that modulate the signaling cascade and especially the Gli transcription factors. Signaling pathways and factors that have been reported to activate Hh ligand expression, including Shh, are EGF induced PI3K/Akt [314], Ras [315,316], NF κ B pathway [317,318], p63 overexpression [319], ROR α via β -catenin and p300 [320], and ER α activation [321]. Hh signaling and Gli protein activity is regulated and modulated by several other signaling pathways and factors, including RAS or RAS/MEK/AKT [322–324], PI3K/Akt [325], PKC δ /MEK-1 [326], JUN [327], EGF/EGFR [328], Notch1 [329], and possibly Wnt/ β -catenin [330]. Hh signaling is reported to downregulate Wnt signaling in gastric cancer through expression of a Wnt signaling inhibitor SFRP-1 [331–333]. GLI1 expression is activated by the transcription factor EWS-FLI1 [334,335] while the protein product of the SCL/TAL1 interrupting locus (SIL) is responsible for the de-repression of GLI1 [336]. The expression of Gli1 and Gli2 is also increased by SMAD3 mediated TGF β signaling [337,338]. Tumor suppressors that negatively regulate Hh signaling are PTEN [323], p53 [339], REN [286,340] and Numb [265]. Active Hh signaling inhibits p53 accumulation [339,341]. In light of the effect oxysterols exert on Smo, it is interesting to note that though OSBP and/or ORP proteins could be involved directly in the Hh pathway, they may also modulate Hh signaling by affecting signaling pathways that interact with the Hh pathway. In addition to regulating shingomyelin synthesis [342] and modulating insulin signaling in hepatic lipogenesis [343], mammalian OSBP also acts as a sterol-responsive regulatory scaffolding protein in the cytosol through its interaction with serine/threonine phosphatase PP2A and the tyrosine phosphatase PTPBS [182], two phosphatases that regulate the activity of the MAPK/MEK targets ERK1 and ERK2. In addition, ORP9 regulates Akt phosphorylation [344], and the oxysterol binding protein BIP in *C. elegans* is involved in modulation of TGF- β signaling [345]. Hh related pathway interactions in tissue repair and carcinogenesis are shown in Figure 7.

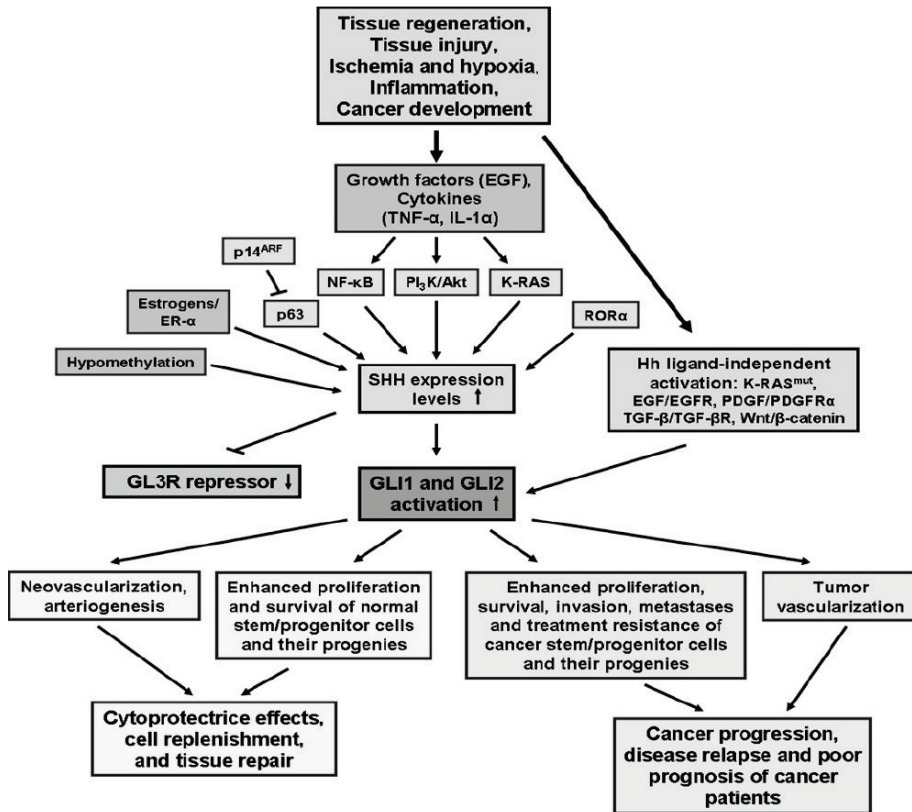


Figure 8. Pathway interaction in tissue repair and carcinogenesis. A schematic representation of cellular events and signaling elements involved in the regulation of SHH expression, Shh dependant and independent GLI activation, and mediation of the Hh activation-induced cellular response. Potential cellular signaling elements involved in the regulation of SHH expression are indicated, including signals activated in response to growth factors and cytokines during tissue injury and regeneration, inflammation and cancer progression. Modified from Mimeault and Batra 2010 [8]

4.5 Hh pathway in disease and cancer

The Hh pathway and proteins required for Hh pathway function are also behind several severe birth defects, disorders and diseases. Mutations that induce loss-of-function in the human *Shh* gene, *SHH*, leads to a loss of the ventral midline and results in a disorder called holoprosencephaly (HPE) [346–349]. HPE phenotypes include cyclopia and impaired separation of the two halves of the forebrain. HPE is found in approximately 1/250 induced abortions and in 1/16,000 live births [350]. Mutations in

PTCH [351] and *GLI2* [352] have also been linked to HPE-like phenotypes. Mutations in *GLI3* are linked to Pallister-Hall syndrome [353,354], Grieg cephalopolysyndactyly syndrome [353,355], Postaxial polydactyly type A [356], Postaxial polydactyly type A/B [357] and Preaxial polydactyly type-IV [357]. The autosomal recessive Smith-Lemli-Opitz syndrome (SLOS) [358,359] results in some phenotypes reminiscent of Hh mutations due to a defect in cholesterol biosynthesis that in turn affects Hh signaling. Bardet-Biedl syndrome [360], Joubert syndrome [361], Kartager syndrome [362] and Polycystic kidney disease [363] are tied to dysformation of cilia and also include phenotypes reminiscent of Hh pathway mutations.

In addition to the disorders resulting from alterations in Hh signaling during development, the pathway is also involved in human disease in young and adult individuals. For instance, activation of the Hh pathway can modulate hair growth [84,364] and erectile dysfunction [365]. But the most prominent human disease involving the Hh pathway is cancer. The first link between the Hh pathway and cancer came when *PTCH* was identified as a tumor suppressor, based on mutations *PTCH* in an uncommon human disorder called nevoid basal cell carcinoma syndrome, or Gorlin syndrome [366–368]. Patients who have the Gorlin syndrome are *PTCH1* heterozygotes, and suffer from birth defects such as skeletal malformations. They frequently develop neoplasia and cancer [369,370] due to mutations that inactivate the remaining wild type allele of *PTCH1*. The *PTCH* heterozygosis predisposes patients with the Gorlin syndrome to various forms of cancers, including multiple basal cell carcinomas, medulloblastomas, ovarian fibromas, and with a lower frequency of occurrence rhabdomyosarcomas, meningiomas, fibrosarcomas and cardiac fibromas [370–372]. *PTCH1* mutations have also been found to be associated with a number of sporadic tumors, and are found in up to 40% or more of sporadic basal cell carcinomas (BCC) [373–376], the most common form of human cancer. BCC represents 1 out of 3 diagnosed tumors, and affects one out of six individuals in their lifetime [377]. Other sporadic cancers include medulloblastoma [376,378–380], squamous cell esophageal carcinoma [381], meningioma [382], transitional cell bladder carcinoma [383], benign skin trichoepitheliomas [384], and various noninflammatory cysts [385]. BCC is believed to be derived from stem cells in the bulge of the hair follicle, cells that have endogenous Hh signaling, and the various tumors mentioned above also arise from tissues that harbor endogenous Hh signaling. In addition to the inhibiting mutations in *PTCH1*, activating mutations in *SMO* have been found to be associated with medulloblastoma [386,387], as well as being identified in about 20% of sporadic BCC's [386–388]. In addition, inactivating mutations in the *SUFU* gene have also been identified in BCC [389]. As *PTCH1* and *SMO* mutations lead to *GLI1* expression, it is not surprising that high *GLI1* expression is a consistent feature of

tumors harboring such mutations [390,391]. This implies that the activity of GLI1, through its transcriptional targets, may be an important or prerequisite step for cancer development in the cells harboring *PTCH1* and *SMO* mutations. Because GLI1 up-regulation is a consistent finding in all BCC's, transcriptional changes would seem to be the most likely mechanism by which the pathway affects tumorigenesis. The *GLI1* gene was in fact discovered due to its gene amplification and high expression in gliomas [392]. *GLI1* gene amplification has been reported in some lymphomas, and sarcomas [393,394], and the degree of *GLI1* gene expression in sarcomas carrying *GLI1* amplification correlates with tumor pathology [395].

In addition to the named tumors where Hh signaling appears to play a central role, abnormal Hh pathway activity has been implied in a broad spectrum of other human tumors, including brain [376,378–380,392], skin [366,367], liver [396], blood (leukemia) [397], lung [81], ovarian [398,399], breast [400–402], bladder [403], prostate [404,405] and gastrointestinal cancers [406] such as oral [407], esophageal, gastric, biliary tract [406] and pancreatic cancer [408]. In many of these tumors however, the cause of Hh pathway activation is not due to pathway mutations, but rather due to over expression of Hh ligands, and especially Shh [81,406,409]. There has been some controversy whether tumor derived Hh ligands act on the tumor by autocrine signaling or on the stroma by paracrine signaling, but the current understanding is that Hh ligands expressed by tumors can act by both paracrine [410–417] and autocrine mechanisms [81,82,406,408]. The involvement of Hh signaling in carcinogenesis is well documented [399,401,402,411–413,416–419]. For instance, Shh acts at multiple stages during pancreatic carcinogenesis [420], and Hh activation is found in both early tumors and metastatic cancers in the pancreas and esophagus [410,421–423]. Transgenic mice with pancreatic specific expression of SHH or GLI2 develop pancreatic tumors [315,408], and similarly, in gastric and prostate cancers, Hh signal activation is associated with cancer progression [82,404,405,424,425]. The involvement of Hh signaling and GLI transcription factors in proliferation in a developmental context supports the idea that abnormal Hh activation may drive tumor cell proliferation. The relation between Hh activation in tumor cells and proliferation is very clear in BCC, and MB. For instance, the expression of an active Smo mutant, SmoM2 in mouse cerebellar granule neuron precursors results in formation of medulloblastoma [426]. There are also some reports that Hh signaling is involved in the maintenance of putative tumor progenitor cell proliferations [427,428] and possibly “cancer stem cells” [77,397,429–435]. In addition to its involvement in carcinogenesis, Hh signaling has also been coupled to tumor metastasis and invasiveness [82,323,425,436], and resistance to chemotherapy and radiotherapy [437–439]. One of the mechanisms for how Hh signaling is thought to be involved in chemotherapy

resistance is the stromal barrier around the tumor tissue resulting from paracrine signaling between tumor and stromal cells [437,440,441]. In support of this, a Hh inhibitor enhanced the delivery of chemotherapy in a mouse model of pancreatic cancer by modulating the vascularization of the stromal barrier [437]. Similarly, cyclopamine treatment led to increased cytotoxic effects of radiation and paclitaxel [442] and inhibition of metastasis in combination with gemcitabine [436]. Shh has also been implied in regulation of transmembrane drug transport, providing a possible explanation to the effect of combined therapy of Hh inhibition and chemotherapy [438,443]. While oxysterols have been found to play an important role in mediating Hh signaling, oxysterols have pro-oxidation and pro-inflammatory properties and may be involved in carcinogenesis [444].

While it is evident that Hh signaling has an important role in carcinogenesis and tumor development, elucidating this role is complicated due to the involvement of additional signal pathways that affect and are affected by the Hh signaling pathway. A detailed genetic study of 24 human pancreatic cancers revealed an average of 64 genetic alterations, and most of these were point mutations. The alterations were found in a set of 12 core cellular signaling pathways and processes that were genetically altered in 67-100% of the tumors [445]. In all of the 24 pancreatic tumors that were tested the researchers found alterations in the KRAS, Wnt/Notch, Hh and TGF β signaling pathways [445], pathways that are important in development and stem cell maintenance. In this setting it is difficult to name one of the pathways the main culprit; as the combined dysregulation of these core pathways may be a major feature of pancreatic tumorigenesis. In addition, the crosstalk between these core pathways is extensive, as discussed above, with one example being the deregulation of Ras signaling that leads to Shh expression and Hh pathway activation in pancreatic carcinogenesis [315].

4.6 Natural and synthetic modulators of Hh signaling

The first natural Hh pathway inhibitor that was identified, cyclopamine, has a very interesting history. In 1957 the United States Department of Agriculture started an investigation of a strange epidemic of cyclopic birth defects in sheep located in Idaho. Researchers found that the fatal birth defects were caused by a teratogen from the plant *Veratrum californicum* [446,447] (Figure 9) that had been indigested by ewes during the gestation period. Based on plant extracts, a steroid alkaloid was identified as the cause of the fatal birth defects, and it was named cyclopamine due to the cyclopia it induced in the lambs [448–451].



Figure 9. *Veratrum californicum* at Silver Lake, Wasatch Mountains, Utah, identified by locals as Skunk Cabbage. In addition to the common name Skunk Cabbage, *Veratrum californicum* is also commonly called “Corn Lily” and “False Hellebore”. It has also been called *Veratrum tenuipetalum*.

Evidence emerged that linked mutations in the Hh pathway genes to cyclopia [346,347,452], and shortly after the Hh pathway was identified as the biological target of cyclopamine [453,454]. In addition to cyclopamine and jervine, this plant also contains several toxic alkaloids [455,456], with veratridine as one example. It is interesting to note that *V. californicum*, and related plants have played a part of natural medicine among both Native Americans and medieval Europeans. The juice of the roots was used to poison arrows before combat, the powdered root was used as an insecticide [457], and plant decoctions were used by both men and woman as a contraceptive [458–460], were used to treat tumors in combination with another plant, *Salvia doriai*, and its teratogenic effect was known to the native Americans [457]. The plant has also been used to treat cold, infections, injuries and pains of various kinds. Among the Shoshone a decoction of the root was taken daily for three weeks to produce permanent sterility in woman [458,461,462]. The white false hellebore, *Veratrum album*, a European relative of *V. californicum* is also toxic and has been used in similar manners [463]. In China, a relative, *V. unigram* has been used as a last resort emetic (induction of vomiting) and

expectorants (as a mucus thinner and cough medicine), and externally as insecticides [462,464]. As the highly toxic alkaloids are produced in growing plants of the *Veratrum* species, the Native Americans harvested the plant during its dormant stage in the winter when using it as a medicine. The levels of toxic constituents are lowest during the winter months when the toxic alkaloids are degraded and metabolized. Cyclopamine has been used extensively for *in vitro* cancer research and in several *in vivo* xenograft models with varying results. Oral cyclopamine can block the growth of UV induced BCC's in Ptch+/- mice by 50%, as well as inhibiting the formation of new tumors [465]. Cyclopamine also reduces medulloblastoma development in Ptch+/- mice [466], and reduces growth of many cancer cell lines in nu/nu mice [82,323,408,467]. Yet cyclopamine can also cause cell death at doses that do not inhibit Hh signaling [413]. Cyclopamine has been stated to convert to veratramine in acidic conditions [468] and is poorly soluble in water. In treatment of gastric tumors, cyclopamine had a better effect when co treatment with antacid was given [469], consistent with the idea of cyclopamine being unstable in acid.

Several derivatives of cyclopamine that have a higher potency, reduced instability and improved pharmacokinetic profiles have been identified, such as KAAD-cyclopamine [470], IPI-609 [421], IPI-926 [471] and cyclopamine tartarate salt (Cyc-T) [472]. In addition to cyclopamine, other plant derived natural inhibitors of Hh/Gli induced transcription have also been identified [6,17]. These include pentacyclic triterpenes, such as betulinic acid, that inhibit Hh/Gli signaling [17] and sesquiterpenes like zerumbone, bisindole alkaloids like staurosporinone and arcyliaflavin C, and physalin B [6]. The natural and dietary alkaloids solasodine, solanidine, tomatidine also act as Hh inhibitors, but with a lower potency than cyclopamine [23]. These compounds are found in plants of the genus *Solanum*, including tomato and potato plants. Natural Hh inhibitors and synthetic Cyclopamine derivatives are shown in Figure 10.

Current Hh pathway antagonists and agonists can be classified according to what level in the pathway they modulate; at the level of Hh/Ptch interaction, at the level of Smo translocation and activation and at the level of Gli activation, nuclear translocation and transcription. Modulators acting at the level of Hh/Ptch interaction are the Hh inhibitor robotnikinin [473] and the Shh neutralizing antibody 5E1 [474].

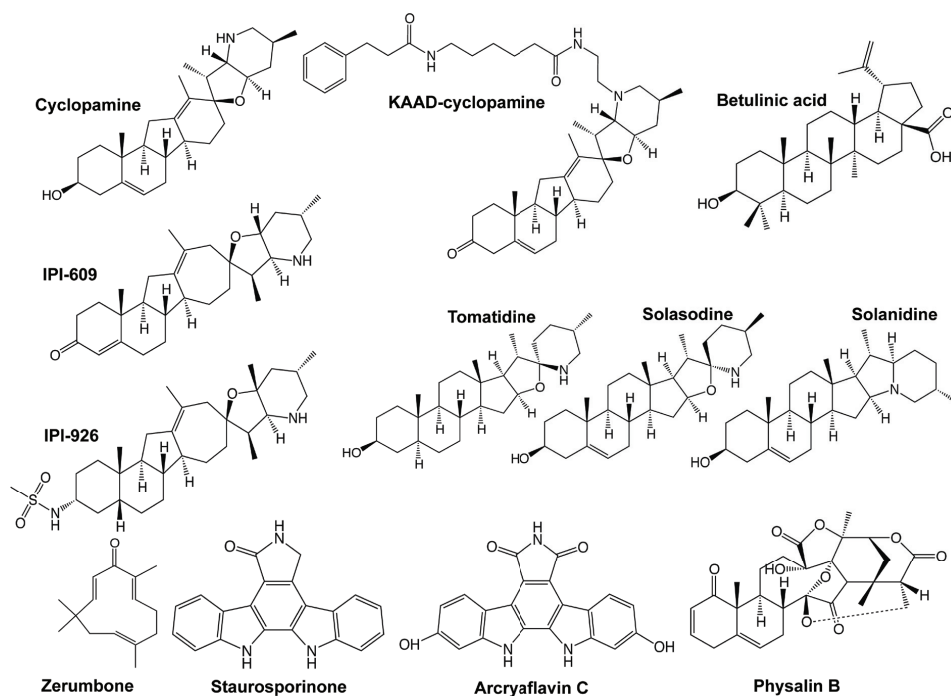


Figure 10. The structures of natural Hh inhibitors cyclopamine, betulinic acid, tomatidine, solasodine, solanidine, zerumbone, staurosporinone, arcryaflavin C and physalin are shown. The structures of the synthetic cyclopamine derivatives KAAD-cyclopamine, IPI-609 and IPI926 are also shown. Modified from Hosoya et al. 2008 [6], Arai et al. 2008 [17], Lipinski et al. 2007 [23] and Heretsch et al. 2010 [5].

Antagonists acting at the level of Smo include cyclopamine [453], KAAD-cyclopamine [470], IPI-609 [421], IPI-926 (Saridegib) [471], provitamin D3 [157], CUR-61414 (HhAntag) [475,476], GDC-0449 (Vismodegib/Erivedge) [477–479], compound 5 and Z [480], 2-amino-thiazole [24], Sant1-4 [14], Sant-75 [481], itraconazole [482], BMS-833923 (XL-139) (<http://www.exelixis.com/pipeline/xl139>), PF-04449913 [483], LDE-225 (Erismodegib) [484] and desmethylveramiline [485]. In addition inhibitors of cholesterol synthesis, such as statins [160], affect signaling between Ptch and Smo. Compounds that activate the hedgehog pathway by agonistic action on Smo are SAG [14], purmorphamine [486] and certain oxysterols [155,156].

Antagonists acting at the level of the Gli transcription factors are HPI1-4 [26], GANT58 and GANT61 [20], arsenic trioxide [487,488], and the natural inhibitors including physalin B that was mentioned above [6]. There are also compounds can inhibit Hh signaling through by targeting signal pathways that modulate Hh signaling. For

instance, the alcohol dehydrogenase 7 (Adh7) inhibitor JK-184 inhibits Gli-transcription factor activity in a dose-dependent manner [489], and Forskolin inhibits Hh signaling by activating PKA that in turn inhibits Gli transcription factors [490,491]. The TGF- β inhibitor SB-431542 blocks TGF- β induced Gli2 transcriptional activity. Structures of Hh pathway antagonists are shown in Figure 11 and 12.

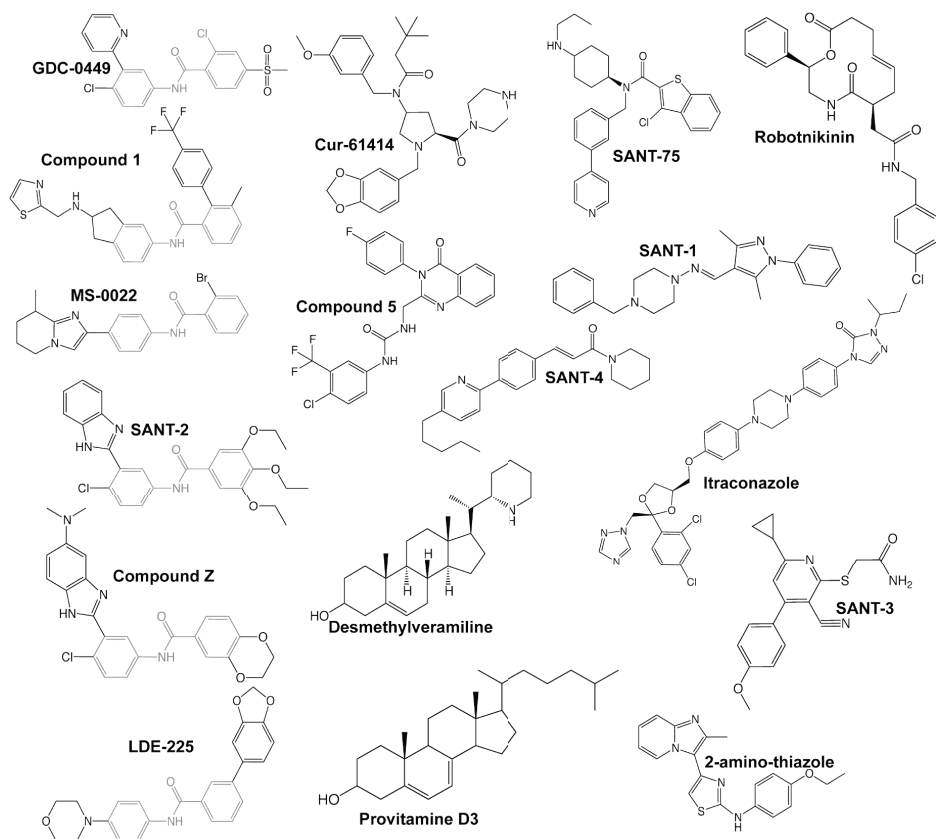


Figure 11. Structures of Hh pathway antagonists. While robotnikinin inhibits the Hh protein, the rest of the compounds are antagonists that block Smo function. Modified from Heretsch et al. 2010 [5] and Peukert and Miller-Moslin, 2010 [13].

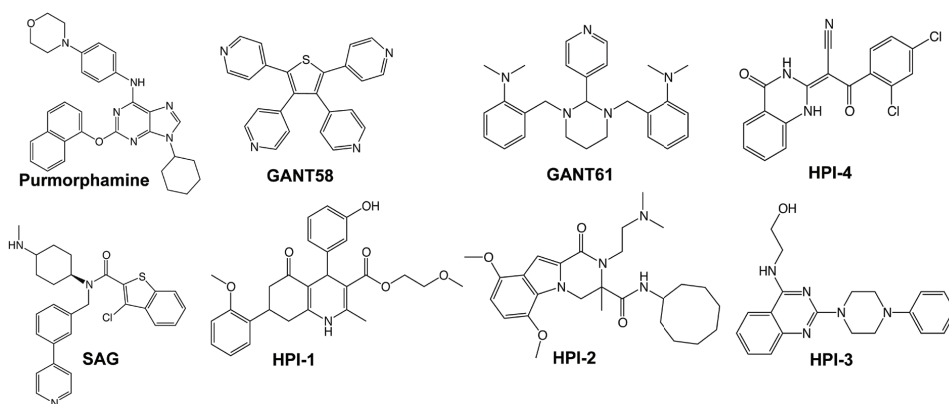


Figure 12. The structures of Hh pathway agonists purmorphamine and SAG and structures of Gli antagonists GANT58, GANT61 and HPI-1-4. Modified from Heretsch et al. 2010 [5], Chen et al. 2002 [14], Lauth et al. 2007 [20] and Hyman et al. 2009 [26].

4.7 Clinical trials with Hh inhibitors

The Roche/Genentech compound GDC-0449, discovered by Curis, and now named Vismodegib (trade name Erivedge) represents the first Hedgehog signaling pathway antagonist to gain U.S. Food and Drug Administration (FDA) approval (January 30th 2012). Erivedge is a Smo antagonist, and is also the first drug approved for treatment of advanced metastatic BCC by the FDA. The drug is intended for patients with locally advanced BCC who are not candidates for surgery or radiation, and whose cancer has spread to other parts of the body. Erivedge has undergone a Phase I and a Phase II clinical trial for BCC treatment [478] prior to the FDA approval, and is also undergoing several clinical trials for diverse cancers, including metastatic colorectal cancer, advanced ovarian cancer, small-cell lung cancer, advanced stomach cancer, pancreatic cancer, medulloblastoma and chondrosarcoma. The phase I trial for BCC treatment included 33 patients, 18 with metastatic BCC and 15 with locally advanced BCC. Of the 18 patients, 8 had confirmed partial responses, 1 patient had an unconfirmed partial response, 7 patients had stable disease as best response and 2 patients had progressive disease, and the overall response rate was 50%. In the 15 patients, 2 had complete clinical responses, 7 patients had partial responses, 4 patients had stable disease as the best response and 2 patients had progressive disease, with an overall response rate of 60%. Erivedge displayed a favorable pharmacokinetic (PK) and pharmacodynamic (PD) profile, and no grade 5 adverse events were observed. The most common side effects in Erivedge treated patients include muscle spasms, hair loss, weight loss, nausea, diarrhea, fatigue, distorted

sense of taste, decreased appetite, constipation, vomiting and loss of taste. The clinical data from Erivedge treatment demonstrates that Hh inhibitors can be used to improve cancer treatment. As an example a case report from the treatment of a medulloblastoma patient can be highlighted. In April 2008 a 26-year-old male patient with widespread systemic metastatic medulloblastoma that was refractory to multiple therapies began treatment with Erivedge. By June the same year the patient's pain score had been reduced from 8 to 0 on a 10-point scale, and the tumors had regressed rapidly, despite the heavy tumor burden and the extent of metastasis at the beginning of the treatment. This regression was transient, however, with a regrowth detected in July, and after a rapid progression of the disease the patient died in September, the same year [492]. Further studies showed that a mutation causing an amino acid substitution in the Smo protein had rendered the cancer cells resistant to the Erivedge treatment [414].

The Infinity Pharmaceuticals compound IPI-926 (Saridegib) is a semi-synthetic cyclopamine derivative [471]. Saridegib is synthesized based on cyclopamine extracted from harvested *V. californicum*, and is currently undergoing clinical trials for the treatment of various types of cancer, including myelofibrosis and chondrosarcoma. Infinity recently ended a Phase 1b/2 clinical trial where patients with previously untreated metastatic pancreatic cancer had been treated with a combination of Saridegib and Gemcitabine. In the Phase 1b portion of the study, partial responses were observed in 5 of 16, or 31%, of patients treated with three different dose levels of Saridegib (Infinity Pharmaceuticals press release). The Phase 2 double blind, randomized, placebo controlled trial comparing a Saridegib/Gemcitabine combination to placebo/Gemcitabine treatment was ended as preliminary data showed a difference in survival favoring the placebo/Gemcitabine arm due to a higher rate of progressive disease in the Saridegib/Gemcitabine arm of the study.

Other Hh antagonists that are under evaluation in clinical trials, but that have not progressed as far as Erivedge and Saridegib, include the Novartis compound LDE-225/Erismodegib and LEQ506, the Exelixis/Bristol-Myers Squibb's BMS-833923 (XL139), the Millennium Pharmaceuticals's TAK-441, and Pfizer's PF-04449913. In addition the Maastricht University Medical Center is performing a clinical trial using Vitamin D3 (from a search in March 2012; <http://www.clinicaltrials.gov/ct2/results?term=Hedgehog>).

5. Aims of the study

The Hh signaling pathway is a key regulatory pathway involved in human development and disease. Abnormal activation of this pathway due to mutations or overexpression has been identified in several human cancers. Many natural and synthetic inhibitors of this pathway have been identified, including the plant derived cyclopamine, and while some of these inhibitors have progressed to clinical trials, one of them have been approved for advanced BCC treatment by the FDA. While it is known that oxysterols and sterol metabolism are involved Hh signaling, the mode of action of the oxysterols in relation to the proteins Ptch and Smo are not fully understood. Reliable identification of specific oxysterols is challenging in biological samples, and especially in the small cellular samples used in standard *in vitro* cell based assays.

The aim of this thesis has been divided into three core aspects; a characterization of a new Hh antagonist, a study of natural Hh antagonists and a study of Hh pathway mechanisms with a focus on oxysterols. These focus areas can be divided into these aims:

- Identify cyclopamine in *Veratrum album*.
- Analyze the weakness and strengths of cyclopamine as an Hh inhibitor.
- Characterize a novel SMO antagonist, and investigate the *in vitro* and *in vivo* properties of this agonist against tumor growth.
- Develop a reliable technique for identifying and detecting oxysterols in small cellular samples, and use this technique to further elucidate the role of oxysterols in the Hh pathway and cancer.

6. Summary of results

6.1 Paper I

Based on the knowledge that the native population in America and Europe has used plants of the *Veratrum* family extensively in natural medicine, we proceeded to investigate *Veratrum album*, a species growing Norway, for the presence of cyclopamine. Using dried benzene extracts from the root of a subspecies of *V. album* (ssp. *Virescens* Gaudin) we investigated whether it could inhibit Hh pathway activity *in vitro*. In a standard Hh assay, using Shh-Light2 cells treated with Shh conditioned medium, we found that the extracts had a 50% inhibitory effect at a dilution of 1:50,000. The initial Mass spectrometry (MS) analysis revealed substantial amounts of what appeared to be cyclopamine, but some of the MS/MS fragments that were seen when analyzing control samples of commercially available cyclopamine was lacking in the analysis of the extracts. This was first believed to be due to performance variations with the MS instrumentation, but when the samples were analyzed on a shallower liquid chromatography (LC) gradient, it was evident that the compound derived from *V. album* ssp. *viriscens* had a shorter retention time than cyclopamine. To confirm these findings, the plant extracts were spiked with cyclopamine, and the analysis revealed two distinct peaks. As this unknown compound had the same mass, and a very similar retention time when compared to cyclopamine, we believed that what we had detected in the plant extracts was an isomer of cyclopamine. The shorter retention time in the reverse phase LC indicated that the potential cyclopamine isomer had slightly less hydrophobic properties when compared to cyclopamine, possibly due to the presence of an additional hydrophilic functional group. The compound was isolated for further elucidation of its structure, properties and biological activity. The compound was diluted with D₂O, and analysis revealed that the deuterated compound had a 1 Da higher mass than deuterated cyclopamine, implying that the compound had an extra hydroxyl group compared to cyclopamine. ¹³C NMR data supported the presence of a hydroxyl group, and the presence of an aromatic ring was confirmed by UV spectroscopy. The presence of the aromatic ring corresponded with the limited MS/MS fragmentation in the isomer compared with cyclopamine. As cyclopamine has been previously reported to convert into veratramine, and as veratramine contains an aromatic ring, the ¹³C NMR data of veratramine and the isolated compound was compared. Only six aromatic resonances were found in the compound while eight was present in veratramine. Based on the interpretation of different NMR spectrums (one dimensional ¹H, ¹³C APT, HSC, HMBC

and NOESY), the isolated compound was identified as dihydroveratramine (DHV). To our knowledge, DHV has not previously been reported as a natural product. We are confident that DHV was present in the plant, and that it was not a product of reduction of veratramine during sample preparation. DHV was detected by LC-MS directly in the crude benzene extract, and a reduction of the double bond in veratramine would be an energetically non-favorable process under the conditions of the isolation procedure. The isolated DHV inhibited Hh signaling with an IC₅₀ of 8 μ M in Shh treated cells and an IC₅₀ > 10 μ M in SAG treated cells. In comparison, cyclopamine had an IC₅₀ of 0,3 μ M in Shh treated cells and an IC₅₀ of 2 μ M in SAG treated cells. Approximately 4% (w/w) of the benzene extract was measured to be DHV. We have demonstrated that VA does not contain cyclopamine, but DHV, a molecule that is easily mistaken for cyclopamine. Both of these molecules have the same molecular formula and mass and similar retention times. The mass fragmentation spectra for these molecules are similar, and the main fragmentation product is the same for both molecules.

6.2 Paper II

In light of the cyclopamine isomer identified in *V. album* and the reported acid instability in cyclopamine, a sample of cyclopamine was exposed to an acidic environment, and selected ion monitoring (SIM) and total ion current (TIC) chromatograms of acidified cyclopamine were generated using capillary LC-TOF-MS. A steep LC gradient similar to one used for PK studies of cyclopamine was employed, and cyclopamine was eluted within a relatively short time (6.5 min) and a single peak appeared in the generated chromatogram. In order to improve the resolution a shallower LC gradient was also used, and two partially co-eluting peaks with the same m/z value as cyclopamine appeared for the acid treated cyclopamine. It was believed that the two peaks were acid induced isomers of cyclopamine. The peak area of these isomers constituted about 40% of the total peak area. It was assumed that the peak area ratio was the actual ratio, and the ratio of the two peaks was not altered within a period of 24 hours, or when the solution was neutralized or basified. In contrast cyclopamine that had not been exposed to an acidic environment appeared as a single peak using the shallower gradient. The isomerization was not seen in non-aqueous, acidic solutions. After 5 days in an aqueous environment a slow reversion was observed, and approximately 1/5 of the peak area of the isomers reverted to cyclopamine. In order to investigate if cyclopamine would isomerize when exposed to stomach acid *in vivo*, we analyzed plasma from mice that had received cyclopamine by PO administration. Isomers were present in the plasma 4 h after administration, demonstrating that exposure to stomach acid will cause

structural alterations in cyclopamine. It is likely that the *in vivo* detected isomers are the same as those detected *in vitro*, but an elucidation of the isomers in the plasma by NMR was not possible. The isomers were not detected in the plasma when cyclopamine was given intraperitoneally (IP), supporting the idea of a stomach acid induced isomerization. As previous reports suggest that cyclopamine converts to veratramine in an acidic environment, the mouse plasma samples were also examined for the presence of veratramine, but no peak corresponding to veratramine was detected. The isomers were extracted from an acidic cyclopamine solution using a preparative LC-UV method. In this preparative method the isomers co-eluted completely. The structure of the isomers in this mix was determined using NMR and computational techniques, and the identified isomers were named cyclopamine (S) and cyclopamine (X). Cyclopamine (X) was less abundant relative to (S) (0.67:1) in the mixture, and no regular cyclopamine was detected. Therefore, it is assumed that the smallest of the partially co-eluting peaks in the LC-MS chromatogram is cyclopamine (X). The (S) isomer has an inverted configuration at carbon atom 23 as compared to cyclopamine, which has R configuration at this position. Quantum chemical calculations confirmed the NMR signal assignment and helped to rule out other possible isomerization products by comparing the calculated and the experimental ^{13}C chemical shift spectra. Using Shh-Light2 hedgehog reporter cells, we investigated whether cyclopamine (S) and (X), retained cyclopamine's antagonistic effect on the Hh pathway. Cyclopamine inhibited Hh pathway activation in Shh induced Shh-Light2 cells by 75% at a dose of 1 μM . Contrasting this effect the extracted isomer mix did not display a statistically significant antagonistic effect to Hh signaling at a concentration of 1 μM . This was confirmed in a dose response assay where natural cyclopamine had a classical S shaped drop in activity and a relative IC_{50} of 250 nM. In contrast, a non-S shaped gradual drop of Hh pathway activity was exhibited by the combined cyclopamine isomers (S) and (X) with a relative IC_{50} of 2.25 μM . Thus the antagonistic activity of the isomers is an order of magnitude lower than seen with natural cyclopamine. It was not determined whether the weak activity that was detected was due to the isomers or whether it was due to a partial reversion to cyclopamine. As cyclopamine (S) and (X) does not block Hh signaling effectively, we investigated at which stage of the Hh pathway the inhibition is lost. Even through natural cyclopamine blocks Hh signaling by binding and inactivating Smo, it also induces the transport of this inactive form of Smo into the primary cilium where it is enriched. Thus, when Shh-Light-2 cells were treated with either Shh or 1 μM natural cyclopamine, increased ciliary presence of Smo was observed as expected. The isomers lacked this activity, demonstrating the absence of bioactive potential. In conclusion, pH-induced cyclopamine isomerization abolishes the potential of the molecule to inhibit Hh signaling at the level

of SMO, and provides a mechanistic understanding for the reduced efficiency of natural cyclopamine in an acidic environment.

6.3 Paper III

A compound (2-bromo-N-(4-(8-methylimidazo[1,2-a]pyridin-2-yl)phenyl)-benzamide) with an IC₅₀ of 100 nM in Shh-L2 cells was identified and named MS-0022. In comparison, cyclopamine had an IC₅₀ of 210 nM whereas GDC-0449 had an IC₅₀ of 30 nM using the same conditions. The structure-activity relationship of MS-0022 was explored using two sets of closely related MS-0022 analogs. All of the analogs had a lower activity than MS-0022, and while some structural alterations led to large drops in activity, some alterations were tolerated (see Paper III for a full description). MS-0022 was shown to compete with BODIPY-Cyclopamine for binding to Smo (IC₅₀ = 259 nM), and effectively inhibited Hh signaling in Shh-Light2 cells activated with Shh, SAG and activating oxysterols (OHC-20 α and OHC-22S in combination). In addition MS-0022 reduced Gli1 mRNA expression in the mouse embryonic fibroblast (MEF) cell line Ptch^{-/-} (based on Ptch^{-/-} knockout mice). While cyclopamine induced translocation of inactive Smo to the cilia, MS-0022 blocked ciliary accumulation of Smo similar to GDC-0449. In contrast to cyclopamine and GDC-0449, MS-0022 reduced Gli1 mRNA expression by 50% in Sufu^{-/-} MEF cells at a dose of 10 μ M. MS-0022 did not block the activity induced by Gli1 and Gli2 overexpression, but effectively blocked the effect of Shh stimulation on Gli1 and Gli2 overexpressing cells. At a dose of 20 μ M, MS-0022 did not reduce nuclear Gli1 translocation in Sufu^{-/-} cells, but the total level of Gli1 in the cells was reduced, correlating well with the reduction in Gli1 mRNA. MS-0022 reduced growth and Gli1 mRNA expression in cancer cell lines PANC-1, SUIT-2, PC-3 and FEMX in a varying degree, and at lower doses the growth inhibition and reduction in Gli1 mRNA did not correlate for all of the cell lines. However, at a dose of 10 μ M MS-0022, the reduction in Gli1 mRNA expression correlated with growth reduction across the four cell lines, compared to a control cell line. This is consistent with an additional Hh pathway inhibitory effect of MS-0022 downstream of Smo/Sufu that requires relative higher doses of the compound as compared to direct Smo inhibition. MS-0022 reduced growth from 40% to 70% in these four cell lines at 10 μ M, while GDC-0449 and cyclopamine had a maximum of 30% growth inhibition at the same concentration. MS-0022 reduced the number of large and medium sized colonies in a soft agar colony assay in a dose dependent manner. While the number of large and medium size colonies decreased, the number of small colonies after MS-0022 treatment increased, indicating that the reduced growth is due to decreased proliferation rather than apoptosis. Long-term

growth assays further confirmed the efficacy of this compound. MS-0022 did not significantly block Wnt signaling or NFkB signaling at doses up to 20 μ M, indicating pathway specificity towards the Hh pathway. MS-0022 led to a slight increase of NFkB signaling, but did not inhibit the activity of any of a panel of 58 Kinases (Millipore diversity panel).

The PO availability of MS-0022 as calculated to be 98% and the half-life in mouse plasma was 55-60 minutes, indicating a moderate stability. The compound was well tolerated in mice upon IP administration of 200 mg/kg for 5 days, and IP administration of 50 mg/kg for 30 days. The animals did not show any outward signs of toxicity or other side effects (weight loss, fur ruffling, hunched posture). Upon the longer exposure, the AST/ALT levels remained greater than 1. PANC-1 xenografts were established in mice and the animals were treated for 30 days with control solvent or 50 mg/kg MS-0022 IP in a 5-day injection/2-day pause scheme. At the end of the treatment there was a 38% reduction in tumor volume in MS-treated animals compared to the control, but the growth appeared to be delayed transiently as the growth curves had the same shape towards the end of the experiment. No reduction in human or mouse Gli1 mRNA expression could be detected at the end of the experiment in the treated tumors compared to the control. In mice xenografted with SUIT-2, similar data was observed. However, after only seven day of MS-0022 PO treatment, Gli1 mRNA expression was reduces in the mouse stromal cells in the tumors. After 18 days of treatment this effect on stromal Gli1 expression was not observed. In the treated SUIT-2 xenografts, similarly to the PANC-1 xenografts, a transient delay of growth was observed during the first days of treatment, followed by a recovery of growth with growth rates in the treated group becoming similar to untreated tumors. Due to substantial variability in the SUIT-2 tumor, no apparent difference between the vascularization of samples derived from treated and untreated animals was detectable. Irrespective of MS-0022 treatment, CD31 staining revealed high vascularization at the tumor edge, while vascularization in the center of the tumor was generally low. In conclusion the identified compound, MS-0022, acts at the level of Smo, blocking its ciliary transport in the nanomolar range. An additional inhibitory effect of on Hh signaling downstream of SuFu that required a dose in the 10–20 micro molar range is linked to a reduction of Gli1 protein levels. MS-0022 transiently inhibited PANC-1 and SUIT-2 tumor growth, and displayed good pharmacokinetic properties and no apparent toxicity.

6.4 Paper IV

The oxysterols that activate the Hh signaling pathway, 20 α -OHC, 22S-OHC, 24S-OHC and 25-OHC have very similar structures. Reliable identification of these, and other similar oxysterols, is very challenging and requires an effective separation step. They share the same molecular mass and mass fragmentation and have very similar logPs. In addition the potential autoxidation of cholesterol present in cells may contaminate oxysterols of cellular origin. After failing to separate oxysterols isomers by a previously established method, a new method was developed that provided the selectivity necessary to separate the isomers with enough resolution for quantitative determination. Under well separating conditions, the oxysterol analytes eluted as rather broad peaks (in both samples and standards), and we speculate that the derivatized oxysterols elute as mixtures of stereoisomers, and this was supported with ^{13}C NMR experiments. Due to their neutral charge, oxysterols are difficult to detect with ESI-MS. Therefore, a “charge-tagging” is appropriate, and this was achieved using a Girard T reagent in large excess. Several challenges were overcome in order to improve oxysterol separation and detection in the development of the described methodology (Paper IV). Cells were lysed/precipitated directly in ice-cold ethanol, pipetted vigorously and frozen. Samples were then evaporated to dryness, and all of the following sample preparation steps were performed in the same tube. The reaction mixture containing tagged oxysterols, excess Girard T reagent, precipitates and particulate cellular matter was injected directly onto the SPE-LC system. An automated filtration/filter backflush (AFFL) technique was used in order to avoid filter clogging, and the loss of oxysterols across the AFFL system was <1%. In comparison, upon spin filtration of the sample before loading into the separation system there was a poor recovery of oxysterols (<1%). Although large amounts of Girard T were injected, the hydrophilic reagent was not retained by the reverse phase SPE enrichment column employed, and was flushed to waste without entering the separation column and the MS instrument. When employing automated AFFL-SPE-LC, only ~10 minutes of total manual effort (i.e. pipetting) per ~20 cell lysates was needed. The method was evaluated for the analytes 22S-OHC, 24S-OHC and 25-OHC, and the range was linear for the standards and spiked cell samples (0.2-33 nM vial concentration, 8-1400 pg injected). Even though 20 α -OHC was initially also monitored, it was not detected in the cell samples tested, and was not included in the final evaluation of the method. The within-day and between-day precisions, calculated as relative standard deviation, for low, medium and high levels of analytes was in the range of 5-19%, and the recovery for spiked lysate samples were 105-109%. In-vial sample intactness was good as samples produced equal peak areas 0-12 hours after first injection. Importantly, in the described methodology, autoxidation during lysis/sample preparation was not an issue for our

analytes, as oxysterol products of a heavy (^{13}C) cholesterol control present in the lysis buffer were monitored for and were for all practical purposes absent. The retention time was constant and the SPE and LC columns held pressures of ~ 50 and ~ 150 bar after the implementation of AFFL-SPE-LC and did not need replacement during the study. As an initial sensitivity test, the oxysterol analytes were assessed in cell lines NIH3T3, Shh-Light2, Ptch $^{-/-}$, Sufu $^{-/-}$, HEK293 (a human embryonic kidney cell line), HCT15 and HCT116 (human colon cancer cell lines). All tested human cell lines contained clearly detectable levels of 22S-OHC, 24S-OHC and 25-OHC, while oxysterol levels in mouse fibroblast cells were significantly lower, with 24S-OHC being under the detection limit in Sufu $^{-/-}$ and NIH3T3 cells, and 22S-OHC being undetectable in Sufu $^{-/-}$ cells. Flasks, vials, solutions and medium were not found to be sources of oxysterol contamination within the method's LOD. In order to identify whether the technique could be used to detect differences in oxysterols between small cell populations, the heterogenic pancreas adenocarcinoma cell line BxPC3 was sorted into vimentin positive (vim+) and negative (vim-), vimentin being a marker for epithelial to mesenchymal transition (EMT). Only 330,000 cells per population were tested by AFFL-SPE-LC-MS ($n=2$ for both vim + and vim -), and the vim+ cells showed clearly higher levels (150 ± 25 pg/100,000 cells) of the oxysterol analyte 24S-OHC compared to vim- cells (58 ± 20 pg/100,000 cells). It remains to be seen whether the detected alterations of 24S-OHC in a subpopulation of BxPC3 cells are of functional relevance, however, the reliability and sensitivity of the here described AFFL-SPE-LC-MS methodology opens for a future detailed analysis of oxysterol levels in small cell populations, and thus will be able to contribute to an improved understanding of this important group of metabolites. As oxysterols are involved in Hh signaling, we exposed a colon cancer cell line, HCT-116 to an Hh antagonist, MS-0022 at $5 \mu\text{M}$. When treated 24 hours prior to lysis, the cells showed a significant ~ 3 -fold decrease of 24S-OHC levels. Although the underlying mechanism behind this effect is unclear, these initial observations encourage further studies.

7. General discussion

In the previous decades, the key aspects of cancer treatment have been the use of resection, radiation, chemotherapy or combinations of these to battle this deadly disease. Both chemotherapy and radiation targets one of the inherent abilities of cancer, the aspect of uncontrolled growth, proving valuable tools in cancer treatment. But where one type of cancer responds well to a certain treatment, other types quickly develop resistance, or re-grow after an initial treatment response. As our understanding of cancer has increased there has been an intensified focus on targeting the signal pathways that the cancer cells depend on for their aberrant abilities. The discovery of the plant derived teratogenic compound cyclopamine was one of several discoveries that played a part in shifting the focus of cancer therapeutics from treating uncontrolled growth, to targeting the abnormal signaling pathways that underlies the uncontrolled growth and other properties of cancer cells. As summarized in the section “aim of this study”, the research summarized in this thesis has focused on three areas related to inhibition of the Hh signal pathway. The first area of focus has been the natural plant derived Hh antagonist cyclopamine, with isomers, combining cell signaling biology with high-resolution analytic chemistry. The second area of focus was the identification and characterization of a Smo antagonist. The third area of focus was on the role of oxysterols in pathway mechanisms and in preparation of this kind of study the development and testing of a method for measuring oxysterols in small cellular samples.

We describe here (Paper I) the identification of dihydroveratramine (DHV) in *V. album* growing in the botanical garden in Oslo, Norway. To our knowledge DHV has not been detected as a natural product previously. However, the synthesis of DHV by reducing veratramine using H₂ gas and a Pt catalyst was reported as early as in 1940 [493]. In addition, DHV was later studied for its activity as a cardiac anti-accelerator [494]. As DHV was detected by LC-MS directly in the crude benzene extract from the plant root, we are confident that this compound was present in the plant and not a product of the isolation process. Also, a reduction of the double bond in veratramine would be an energetically non-favorable process under the conditions of the isolation procedure. We were not able to detect cyclopamine in *V. album* contrary to a previous report [495]. Another subspecies of the plant was used in this study, so we cannot rule out differences between the subspecies of *V. album*. In addition DHV is very easily mistaken for cyclopamine in L-MS analysis. Both of these molecules have the same molecular formula, mass and similar reverse phase retention times. The mass fragmentation spectra for these molecules are also very similar, with the main fragmentation product being the same for both molecules. DHV possessed an *in vitro* Hh inhibitory effect that was about 1

log lower than the activity of cyclopamine, and failed to induce translocation of Smo to the cilium. When comparing the Hh inhibitory action of the crude benzene extracts with the activity of the isolated DHV, it is evident that there may be other compounds in this plant that affect the hedgehog pathway. As the extract inhibited Shh induced signaling stronger than SAG induced signaling, similarly to cyclopamine, the Hh inhibitory activity in the extract likely acts on Smo. A DHV precursor or one or more DHV/cyclopamine related compounds that have a higher potency towards the Hh pathway could be present in *V. album*. While *V. album* has been a part of nature medicine in the past, it is still in use in “alterative” medicine today. In light of the Hh inhibitory activity identified in this plant, the medical use of this plant is highly questionable. Yet, *V. album* is still found in some commercial products. One product containing *V. album*, along with other plants, which has been marketed as an “infantile cholic remedy” has recently (2010) been associated with apparent life threatening events among infants [496].

Issues with poor solubility in water and early reports that cyclopamine converted to veratramine in an acidic environment [497] has led to the development of different derivatives. In contrast to the previous reports we found two previously undetected isomers (S and X) of cyclopamine instead of veratramine after treating cyclopamine with acid (Paper II). The isomers can easily be mistaken for active natural cyclopamine when analyzed by standard LC–MS analysis conditions. In pharmaceutical analysis it is common to use short analysis times, and a steep solvent gradient. However, only when using a slower, shallower (and hence higher resolving separation) gradient it was possible to reveal a large degree of isomerization of natural cyclopamine as a result of acidification. A mechanism for the acid induced isomerization is proposed, including a ring opening and either a cyclization or an elimination of water, and the isomerization is believed to be reversible. The reappearance of original cyclopamine in an aqueous (S/X) solution indicates the existence of a thermodynamic equilibrium between at least two forms. From a chemical point of view the first step in this back reaction, hydratization of one of the double bonds in the conjugated double bond system, seems less likely. So our conclusion at this stage is that the R and S forms of cyclopamine do indeed freely isomerize between themselves in acidic water solution, however the kinetics of this transformation is not fast; chromatography of the isomers the same day after isolation did not show traces of regular cyclopamine. The isomerization appears to occur *in vivo* as isomers were detected in serum samples after per oral administration of cyclopamine in mice. IP administration of cyclopamine did not result in isomerization *in vivo*, supporting the idea of a stomach acid induced isomerization. During the study, our group detected only marginal/less than detectable amounts of veratramine after acidification of cyclopamine. This agrees with an earlier report [495], but is in disagreement with

Keeler [498], where the evidence for the conversion to veratramine is based on IR spectroscopy and thin layer chromatography (TLC). We speculate that the IR spectrum of cyclopamine (X) is similar to that of veratramine, since they both have additional hydroxyl groups and double bonds.

The biological activity of cyclopamine against the Hh pathway is markedly reduced in the acid induced isomers. However, since a major portion of cyclopamine does not isomerize, the remaining “regular” cyclopamine will still be able to inhibit the Hh pathway outside the stomach, as has been confirmed by others [436,465]. On the other hand, it has been reported that cyclopamine is ineffective regarding the Hh pathway in gastric acid [469]. As cyclopamine does not isomerize more than 40% or less in acid, we speculate that it is the protonation of cyclopamine in acid rather than isomerization that causes the drop in effect in a gastric environment. The importance of protonation on a drug has been previously demonstrated [499]. It was not determined whether the weak Hh inhibiting activity displayed by isolated isomers was due to the isomers themselves or due to a partial reversion to cyclopamine when in the aqueous environment of the cell medium. The pharmacokinetics of the isomers is unknown, and whether protonation or isomerization affects uptake of cyclopamine in the digestive tract is not clear. In addition the *in vivo* toxicity or side effects of the isomers are not known. As these isomers have remained undetected in previously reported analysis of cyclopamine, it may be that this is the case for other pharmaceuticals that have been analyzed with similar resolutions. To our knowledge, this is one of the first reports on sterol drugs showing a decrease in activity because of isomerization due to acidification. Thus we show that high-resolution analysis with shallow gradients is critical for detecting isomers that may be overlooked using a steep gradient. The awareness of pH-induced alterations of cyclopamine into nonfunctional isomers/states has an impact on the understanding of its pharmacokinetics and may provide a further basis for the rational design of bioactive analogues. For both natural cyclopamine, and novel analogues, it will be important to detect possible isomers with altered bioactivity, especially if such isomers escape detection in standard analysis.

Our results with detection of a previously unreported isomer of cyclopamine provided an explanation to a phenomenon that was already known. Cyclopamine was less potent or unstable after passing through acid. The implications of this study however stress the importance of high-resolution analysis of drugs; especially as oral drugs are passing through the rough and highly acid environment in the stomach. Drug isomerization in itself is not a new problem, and has been highlighted before due to cases like the thalidomide disaster. Thalidomide was a drug used to treat nausea. In the late

fifties and early sixties over 10.000 children across several countries were born with deformities after thalidomide use by pregnant woman. One of the most common deformities associated with thalidomide use is Phocomelia, where the children were born with “flipper” like limbs. A link between thalidomide and the birth defects was found in 1961 [500,501], and it was later discovered that it was an optical isomer of thalidomide that caused the teratogenicity [502]. The safe thalidomide isomer was shown to convert to its teratogenic enantiomer one in the human body. Drug isomerization is thus a very important issue, especially considering that possible isomers may be overlooked when using the steep gradients commonly used in pharmacokinetic studies in drug discovery.

The compound 2-bromo-N-(4-(8-methylimidazo[1,2-a]pyridin-2-yl)phenyl)-benzamide (MS-0022) was identified as a potent antagonist of Hh signaling (Paper III). Similar to GDC-0449 and cyclopamine, MS-0022 inhibited Hh pathway activity after induction by various agonistic factors like Shh, oxysterols and SAG. The ability of MS-0022 to compete with BODIPY-cyclopamine binding suggests that it acts by a similar mode of action as structurally related Smo antagonists, such as GDC-0449. This was further confirmed by the inhibition of Smo translocation to the primary cilium by MS-0022. MS-0022 contains a core structural motif common to other Smo antagonists. Analysis of the core structure of MS-0022 revealed that the “phenyl-amide-phenyl” portion of the molecule was required for activity, and this core structure is also present in HhAntag [33], GDC-0449 [52], Sant-2 [53] and Compound Z [54] and other structures (see grey core structure, Figure 11). Although the side groups sticking out of the core structure in these molecules differ, their properties are similar. While MS-0022 inhibited Smo in the nanomolar range, it displayed an additional effect on the Hh pathway downstream of Smo that required a higher dose. In the micromolar range, MS-0022 led to a significant reduction of Gli1 mRNA and protein levels in Sufu-/- cells. This effect was not due to an altered or inhibited translocation of Gli1 to the nucleus, indicating that MS-0022 did not reduce Gli1 levels by direct inhibition of Gli1. The precise cause of this secondary effect remains to be elucidated. As described in the introduction section of this thesis, several of the proteins in the Hh pathway have dual roles, and some of the interactions are not properly understood. It should be noted that GDC-0449 has been shown to block ABC class membrane transporters in addition to blocking Smo [503]; and although this was not investigated in this work, it could well be that this is the case for MS-0022 and other Smo antagonists that share similar structures.

As the implications of aberrant Hh signaling are getting increasingly evident in many types of cancer, new pathway antagonists will be an essential part of the toolkit for the development of future treatments. The emerging data from clinical trials with GDC-

0449 show both the benefits and possible pitfalls of a pure Smo antagonist; a clear tumor response in Hh driven tumors such as basal cell carcinoma and medulloblastoma [414,504,505], and the occurrence of a drug induced resistance caused by mutations in the Smo locus [414,504]. Our MS-0022 based research displays that Smo inhibitors are good candidates for Hh inhibition in cancer, but the development of resistance is a challenge. The mutation conferring resistance to GDC-0449 in medulloblastoma cancer cells was mapped to the central binding cavity in GPCR helix bundle of the Smo protein [414]. As several GDC-0449 related (see figure 11, compounds with a grey core structure) and non-related compounds appear to block Smo function, the best way to target Smo in cancer would likely be to exploit this diversity. By identifying Smo antagonists that bind to different parts of Smo, and using these in combined treatment, one could greatly reduce the chance of point mutations leading to treatment resistance. It was demonstrated by Dijkgraaf et al. that cyclopamine, Sant-1 and other Smo antagonists based on the GDC-0449 core structure reduced Hh signaling in tumor cells that were resistant to GDC-0449 [506]. Whether MS-0022 would be active against the GDC-0449 resistant tumor cells have not been determined. Attacking a signaling pathway at two sites at the same time makes sense when taking the high mutation rate in cancer cells into account [506,507]. Thus a mutation rendering the cell resistant to one of the drugs would not have a much greater chance of surviving than the non-resistant cells, and the development two mutations at the same time is a much less likely event. The possibility of such a dual inhibition tactic would also be relevant to consider for other signal pathways that are targeted for cancer treatment. Another possible solution to blocking Hh/GLI1 signaling in patients with a developed resistance to a Smo antagonist would be to develop drugs that act on the pathway downstream of Smo [20]. A Gli inhibitor, for instance, could be used to target tumors harboring non-canonical activation of Gli [508] or mutations that cause activation of the Hh pathway downstream of Smo, in addition to tumors that have developed a resistance to a Smo antagonist. However, in a recent study, point mutations in Smo were detected in only 7 of 135 tumors resistant to the Smo inhibitor NVP-LDE225 [509]. The high level of tumor-stromal interactions found in advanced cancers like pancreatic adenocarcinoma poses additional challenges for developing treatment strategies based on Hh antagonists. Such strategies would require that, in addition to autocrine efficacy, paracrine interactions between tumor activated stroma and tumor cells are disrupted [413]. Furthermore, the barrier that the stromal cells create around the tumor, and the lack of compound delivery to areas of low vascularization needs to be addressed [437].

MS-0022 readily entered the body tissues after per oral administration and was well tolerated *in vivo*. In addition to blocking the growth of several tumor cell lines in

various assays *in vitro*, MS-0022 also inhibited the growth of SUIT-2 and PANC-1 tumors in xenograft experiments. The growth inhibition appeared to be transient, and despite a strong growth inhibition in the early days of the treatment the growth recovered and eventually had the same growth pattern as the untreated tumor cells. Our *in vivo* results also lend support to the idea that the primary effect of Hh inhibition in pancreatic cancer treatment is due to the inhibition of Hh pathway activity in the stromal cells, although we also observe a mild initial reduction of Gli1 levels in tumor cells. The initial growth inhibition correlates with the reduction in stromal Gli1 mRNA levels after 7 days, while after 18 days the Gli1 mRNA levels and growth was no longer blocked by MS-0022. Olive et al reported that treatment with another Hh inhibitor, IPI-926, that also displayed a transient effect in the treated tumors [437]. This study was based on mice that develop pancreatic tumors that resemble human pancreatic ductal adenocarcinoma due to a conditional expression of endogenous mutant Kras and p53 alleles in pancreatic cells (KPC-mice) [316,510]. Both the SUIT-2 and PANC-1 cell lines are mutant for p53 and Kras [511–515]. Although the transient inhibition could possibly be due both to an increased stromal barrier, or be due to mutations that lead to drug resistance, another possibility should also be considered. Hh inhibition may trigger the recruitment of other core signaling pathways to take over the proliferative drive in the tumors, and Smo inhibition could also lead to an uncoupling of Gli from Smo control, allowing other pathways to take over the control of Gli proteins. For instance, upregulation of PI3K signaling has been linked to the development of resistance against a Smo antagonist in cancer, but the underlying mechanism remains to be investigated [509].

Oxysterols have emerged as molecules of interest and have been studied within various fields for the last 30 years. The effect and regulation of Hh active oxysterols are little known, but are of great importance to investigate in order to understand this cancer related pathway in greater detail. As reviewed by George J. Schroepfer Jr. (2000), the analysis of oxysterols is hampered by low physiological concentrations in comparison to cholesterol, autooxidation and difficulties with analytic techniques. Several separation techniques and analytic methods have been used and are in use today for detection of oxysterols. In order to detect the low amounts of oxysterol present, larger amounts of sample material are required. In addition the sample preparation and pre separation and pre-processing of the sample prior to analysis is difficult and time consuming. We report here (Paper IV) a sample preparation technique combined with an AFFL-SPE-LC-MS analysis method that allows the determination of a set of highly Hh active oxysterols using just one sample transfer step (from preparation tube to autosampler vial). Compared to established methodology, the manual sample preparation was significantly reduced. The total hands-on time per 20 lysed cell-samples were about 10 minutes.

Clogging was avoided by using AFFL and excess Girard reagent did not enter the MS instrumentation as it did not bind to the SPE column and was flushed to waste before the elution of analytes. The AFFL-SPE-LC-MS methodology was validated (precision, sensitivity etc.) with good performance. The chromatographic resolution of the oxysterol isomers 22S-OHC, 24S-OHC and 25-OHC was satisfactory for our purposes, and the method was shown to be suitable for determination of the oxysterol analytes in various cell lines with a high degree of precision. The methodology was also reliable for the analysis of relatively small cell samples (330.000 cells), allowing us to study selected cell subpopulations. Thus this methodology could be used on stem cells, on cellular subpopulations or on other cells that are difficult to harvest in higher quantities. We have here focused on determination of oxysterols of known, strong potency regarding the Hedgehog pathway, and the methodology may be expanded in follow up studies to other oxysterol groups and other similar cellular lipophilic metabolites. In addition to the oxysterols involved in the cancer and stem cell related Hh pathway, other oxysterols are for instance involved in atherosclerotic plaque formation [171,516]. 24S-OHC has been suggested as a marker for neurodegenerative diseases like alzheimers disease and multiple sclerosis, as the concentration of this oxysterol is increased in the spinal fluid and decreased in the blood of patients suffering from these diseases [517,518]. One of the main reasons for the development of this methodology has been to find a way to be able to detect oxysterols of interest in cellular samples in the size-range normally used for cellular assays, as current methodology failed at this task in our hands. But we believe our technique could be a valuable tool in measuring oxysterols in body fluids, if they prove to be valid markers of disease. In addition the involvement of oxysterols in carcinogenesis [444], and the high levels of oxysterols we have detected in the colon carcinoma cell lines HCT15 and HCT116 merits further investigation to whether oxysterols could be a marker for cancer development or treatment progression.

The use of this technique have already provided us with a couple of very interesting observations. The biological relevance of these observations needs to be validated further in follow up studies, but they have demonstrated the sensitivity and usefulness of the developed methodology. For instance we were able to measure the oxysterol content of samples from several cultured human and mouse cell lines with very little standard deviation between replicates. The ratio of different oxysterols across the analyzed cell lines varied, and was more similar between cell lines of the same species and cell type. In addition, two oxysterols (24S-OHC and 22S-OHC) present in the *Ptch*^{-/-} MEF cell line were not found in the related *Sufu*^{-/-} MEF cell line. It remains to be shown whether these levels are related to the presence/absence of *Ptch*^{-/-} or if they are a result of differences in morphology or other cellular differences. It could also be speculated that

the Hh pathway in *Sufu*^{-/-} cells is independent on oxysterols as the pathway is constitutively activated below the level of Smo. Another interesting observation is that inhibition of the Hh pathway by MS-0022 reduced the cellular levels of 24S-OHC by 3-fold after 24 hours. Whether this reduction was due to enzymatic depletion or cellular efflux of 24S-OHC remains to be shown. As Ptch has been shown by others to pump cholesterol out of the cell, it is possible that Ptch could pump oxysterols in a similar manner. Another possibility is that the efflux of cholesterol mediated by Ptch could result in lower cholesterol levels and in turn lower production of oxysterols. While some reports claim that 24S-OHC acts as an agonist of LXR [519,520], but a newer *in vivo* study contradicts this claim [521]. 24S-OHC has primarily been identified as a carrier of cholesterol from the brain to the liver. Unlike cholesterol, 24S-OHC can pass the blood/brain barrier, and about 7 mg 24S-OHC is transferred from the brain to the liver in a 24 hour period [522]. As oxysterols can pass cell membranes and bind lipoproteins at a much higher rate than cholesterol, oxysterol formation may be a mechanism utilized by some cells to eliminate excess cholesterol in addition or in stead of cholesterol transport mediated by ABC transporters and HDL [522]. In light of the discovery that GDC-0449 blocks ABC transporters in addition to blocking Smo, the effect of MS-0022 on 24S-OHC may not be Hh pathway related, but rather related to an effect on ABC transporters. The ABC transporter ABCA1, for instance, has been shown to mediate efflux of oxysterols from fibroblast cells [523]. Still, a possible MS-0022 mediated blockage of ABC transporter efflux of cholesterol or sterols does not fit with the decreased 24S-OHC levels detected after MS-0022 treatment. An effect of MS-0022 on ABC transporters would also fail to explain how SAG would lead to an increase in 24S-OHC level in contrast to the reduction mediated by MS-0022. The wide variety of both synthetic and natural lipophilic compounds that bind and modulate Smo activity indicate that Smo might be a more general lipid sensory receptor. Likewise Ptch has been reported to modulate the levels of cholesterol [154] PI4P [163], provitamin D3 [157] and possibly oxysterols. Although it seems that oxysterols activate Smo directly as allosteric effectors [159], they may also modulate the Hh pathway through other mechanisms. Oxysterol binding proteins are involved in vesicle transport, late endosomes, Akt signaling and ERK signaling; all of which are involved in or modulate Hh signaling. It is interesting to note while cholesterol and 25-OHC regulates the OSBP/PP2A/PTPPBS complex and thereby ERK activity, PP2A has been identified as a possible Hh pathway component in an RNAi screen [524]. Although Nachtergaele et al. finds 20S-OHC as the primary oxysterol activating Smo, 22S-OHC and 25-OHC have activated the pathway similar to 20S-OHC in our hands. In our hands 24S-OHC was more potent in activating Hh signaling than the other reported HH activating oxysterols. In addition we have found that all of these oxysterols have a synergistic effect on Hh pathway activity. It could be that

the specific oxysterols target different proteins, or that the binding of different oxysterols to the two parts of a protein dimer enhances the activity. Dimerization has been reported for both OSBP and Smo. The role of PI4P in Smo activation may be to recruit OSBP or ORP proteins to the vicinity of Smo, thus facilitating the transport of oxysterols to activate Smo in absence of Ptch, but this remains to be proven.

In conclusion this thesis has focused on the Hedgehog signaling pathway, an important developmental pathway that is abnormally activated in several forms of cancer, including types of cancer that are difficult to treat. The reported research has encompassed the natural and plant derived Hh inhibitors cyclopamine and DHV, the synthetic Hh inhibitor MS-0022 and cell-derived oxysterols. We have identified DHV in *V. album*, and showed that this compound is a weaker Hh antagonist than cyclopamine. We did, however, not find cyclopamine in the subspecies of *V. album* that we had access to. We have identified two previously unknown isomers of cyclopamine that are formed when cyclopamine is in contact with acid, and showed that these isomers are an order of magnitude less potent antagonists of the Hh pathway than cyclopamine. These results confirm the weakness of cyclopamine in acidic environments. We have also characterized the effect of the Smo antagonist MS-0022 against cancer growth *in vitro* and *in vivo*, finding that although this compound reduced tumor growth in mice xenografts, the effect was transient. Further we have developed and effectively tested a method for detecting oxysterols, an important signal transducer in Hh signaling, in small cellular samples, making observations that merit further studies.

8. Appendix I. Drug discovery, target deconvolution and chemical analysis

The discovery and use of natural and synthetic drugs have had a huge impact in medical science and practice in the last century. In short, the term “drug discovery” encompasses the process by which drugs are identified, tested and developed. The drugs in this context are molecules that modulate biological processes, often by modulating protein activity and function. Drug discovery is an important part in the fields of medical research, biotechnology and pharmacology. The use of drugs is not a new phenomenon in human history. Various plants and herbs have been used as natural remedies in the past and are still being used today. However with the development of the fields of organic chemistry and chemical analytics coupled with a greater understanding of genetics and biochemistry it has become possible to isolate the chemical compounds in a plant that has a biological function, as well as making new compounds that are not found in nature. The number of possible organic compounds that can be made with a molecular weight below 500 Daltons is estimated to be over 10^{60} [525]. In comparison the earth is estimated to be made up of 10^{51} atoms. When looking for compounds that have a specific biological action, it is thus not feasible to synthesize and test the activity of this staggeringly high number of possible compounds. The strategy has instead been to make large libraries of compounds with as diverse chemical structures as possible, and to use a technique called “high-throughput screening” (HTS) to identify compounds that affect a specific biological process. Whereas drug discovery normally begins with HTS, it is followed by a Hit-to-Lead phase where the activity of possible drugs is confirmed, and the best candidates are chosen for further development before animal experiments and a possible clinical trial. Although the scheme of drug discovery is well established it is not an easy process. The cost of the research and development required to produce a “new molecular entity” (a new approved drug that is not similar to a previously approved drug) was estimated to be approximately 1,8 billion USD in 2010 [526].

The use of HTS for identification of compounds that targets a specific biological process requires the development of an assay where the activity of the biological process can be monitored in a large number of samples. Processing large numbers of samples and assays manually is very time consuming; but the use of multi-welled assay plates, automated robotics, control software, computer based data processing, liquid handling devices and sensitive detectors have speeded the process up, enabling a researcher to conduct anything from hundreds to millions of chemical or biological tests in a relatively short timeframe. A wide range of detection techniques are in use, depending on the type

of assay, including various forms of light detection, such as luminescence measurements and microscopy. Computation and statistics can then be used to process the acquired data in order to identify compounds that affected the reaction or process used in the assay. The identified compounds are normally tested in additional screens and assays in order to verify their activity, and to identify the best candidates for further development. Specificity, cytotoxicity, analogging, structure-activity relationship analysis (SAR), deconvolution, target affinity, druglikeness, rational drug design and pharmacokinetic studies are important aspects of drug development and optimization. The term “druglikeness” describes a compound’s potential as a drug with respect to structural factors that influence bioavailability. There are two major approaches to HTS in drug discovery. The more classical approach, termed chemical genetics [527,528], has been to expose cells or organisms to a library of plant derived compound or synthesized chemicals, and to look for cellular or phenotypic changes in order to identify compounds that affect a biological process. As this approach yields hits that affect a system or process, it is challenging to identify the direct mode of action. In order to avoid this problem, the focus shifted to target-based drug discovery. Assays were developed where a specific reaction or a specific protein interaction was assayed against chemical libraries of compounds. The target-based drug discovery has not work as optimally as hoped [529], and the field has now shifted back towards more chemical genetics [527]. Several strategies have been developed that aim to help discover the mode of action of a potential drug that affects a biological process, but this can be a time consuming and difficult research. This determination of a compound’s mode of action is called target deconvolution. When a compound affects a cellular signaling pathway or a biological process has been identified, assays can be designed that will allow the researcher to distinguish at what level of a process or pathway the drug exerts its effect. But even when the level has been identified, target deconvolution can be a challenge. The Hh pathway is a good example where target deconvolution is difficult. Several of the interacting proteins in this signalling pathway have dual roles, and the proteins are part of several different protein complexes where the interactions are not fully understood. Yet, if the drug binds a certain protein, in theory this interaction between drug and protein should be detectable. One approach is the use of affinity chromatography, a technique primarily used for protein purification [530], in identifying cellular protein targets of small drugs [531–533]. The small compound of interest is tagged by introducing a chemical group called a linker. This linker can act to immobilize the compound by binding it to a solid material that is referred to as a matrix. The compound with its attached linker can then be incubated with a protein extract in presence of the matrix, followed by washing steps that will remove all

proteins that do not bind to the matrix-linked compound. After several washing steps the linker-matrix interaction can be disrupted to elute the bound protein for immunodetection [534] or MS analysis [535]. In order to use this technique, the drug of interest must tolerate the attachment of a linker without losing its activity. Another limitation is that a specific compound often can bind several types of proteins. Further steps may be required to distinguish which protein of a group of proteins that interact with the compound that is responsible for the biological activity, while removing “false positives”. Other techniques used in drug discovery are expression cloning technologies such as the yeast three hybrid system, phage display and mRNA display where protein abundance is artificially increased [536], protein microarray, cellular cDNA array, biochemical suppression [536] and anisotropy based analysis. In order to discriminate between positive and false-positive compound-protein interactions, the protein binding targets of a compound can be mapped by biological function using bioinformatics; allowing the researcher to determine which of the proteins is the most likely candidate to be involved in the targeted biological process or pathway [537]. *In silico* computational compound-protein interaction profiles [538,539] may be generated if three-dimensional protein structures are available. Various methods can be used to confirm a physical interaction, including surface plasmon resonance, resonance acoustic profiling and nuclear magnetic resonance (NMR). In order to verify that the compound’s effect is due to an interaction with a certain protein, it is possible to investigate the cellular or biological effect of altering the protein level directly. If the compound is a process or pathway inhibitor or agonist, an RNA interference knockdown of protein expression should have the same phenotype or effect. If the compound acts as an agonist that activates or enhances a pathway or process, overexpression of the identified target protein should display the same effect.

The field of chemical analysis has provided several important tools that are important in drug discovery as well as the research fields of biochemistry and molecular biology in general. Tools that have been important for this thesis and that are widely used in drug discovery are high performance liquid chromatography (HPLC), mass spectrometry (MS) and NMR spectroscopy [540,541]. Chromatography is widely used to separate different substituents of a mixture, both in liquid and gaseous phases. In HPLC, a mixture is pumped with the mobile phase through a column for example containing particles with a stationary phase attached. For high performance (and high resolution) separations, the particles must be small (~2 to 5 μm). This creates a high back-pressure, and that is why HPLC is often called high pressure liquid chromatography. Each of the components in the mixture will be differentially partitioned between the stationary and

the mobile phase, and this will cause them to move through the system at different speeds. This difference is used to separate the constituents of the mixture. The chemical property of the stationary phase that is selected is based on what kinds of substance that is to be separated. The most commonly used technique today is reversed phase HPLC. The more hydrophobic a component is the longer it will take to move (elute) through a reversed phase LC system. This is opposite (reversed) of the first available LC columns, where more hydrophilic compounds elute later (commonly called “normal phased” liquid chromatography). The bioavailability of drugs depends on both hydrophilic and hydrophobic properties, as the drugs have to pass through body fluids and cell membranes. Reversed phase LC is very often used to separate drugs or drug candidates according to their hydrophobic properties. The time it takes for a compound to elute is called the retention time. After separation the different constituents of a mixture can be fractionated for further analysis or be monitored/analyzed in real time by coupling the LC system to a detector. Common detection systems are based on UV absorption spectrometry or MS. MS analysis however requires that the analytes are in gas phase. This initially limited the use of MS to gas chromatography, which is limited to analysis of volatile, thermostable compounds. However, the development of the electrospray ionization (ESI) [542] technique allowed LC to be coupled more readily to MS. In brief, MS analysis is based on ionization of molecules or molecular fragments before measuring the mass-to-charge (m/z) ratio. The MS technique has several uses, including aiding the determination of a compounds structure based on observations of fragmentation, and for identification and highly selective quantification. The ion abundance versus m/z is plotted in a mass spectrum. An MS instrument contains an ionization source, a mass analyzer and a detector. Several different ionization sources and mass analyzers have been developed that have different properties. The purpose of the ionization source is to generate ions (gaseous) that are subsequently sorted by their masses in the mass analyzer by utilization of electromagnetic fields. After ionization and sorting the m/z ratio is measured in the detector. Two key ionization methods are ESI [542] and matrix-assisted laser desorption/ionization (MALDI) [543] and the efforts related to the development of these two techniques lead to a share of the 2002 Nobel Prize in Chemistry.

ESI is, as mentioned above, used to couple LC to MS instrumentation. In brief, ESI is produced when a liquid sample is passed through an electrically charged capillary. The polarity of the voltage applied to the capillary will result in an electrophoretic effect, where the eluent will have either a net positive or a net negative charge. Due to resulting repulsion within the eluent, it forms an aerosol spray of charged droplets (a “Coulomb explosion”). As the droplets pass through air or nitrogen steam the droplets shrink in size

due to evaporation and subsequent Coulomb explosions. Two different theories are used to explain the final stage of ESI, namely the event of ions entering gas phase intact [10].

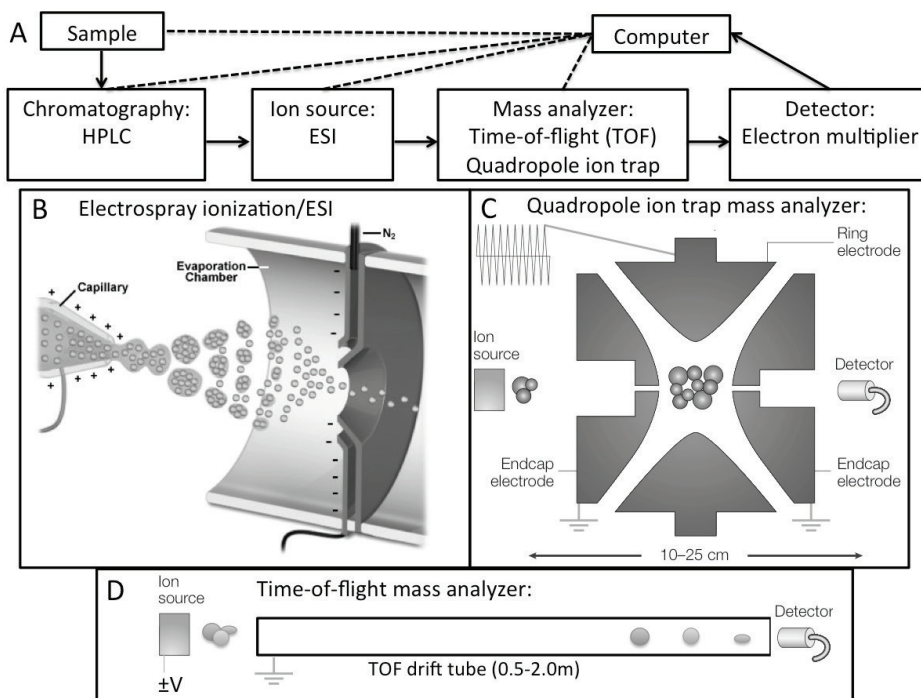


Figure 13. LC-MS instrumentation. A. A schematic depiction of a typical LC-MS setup. B. A figure showing the use of ESI as an ion source. Tiny droplets containing charged analytes are sprayed out of a capillary, and gas phase ions are formed due to solvent evaporation and coulomb explosions in the evaporation chamber. C. The quadrupole ion trap mass analyzer utilizes a structure containing two endcap electrodes and a ring electrode. Electric fields over these electrodes are used to trap ions from the ion source and to release ions into the detector. D. The TOF mass analyzer separates accelerated ions based on m/z ratio as the ions drift through the TOF drift tube. Modified from http://www.magnet.fsu.edu/education/tutorials/tools/ionization_esi.html, Glish et al. 2003 [4] and El-Aneel et al. 2009 [10].

According to the charge residue theory the tiny droplets formed by Coulomb explosions are thought to undergo complete evaporation, thus releasing the charged molecules. In contrast the ion evaporation theory is based on the idea that the ions are desorbed from the surface of the tiny droplets. The gaseous ions formed are then passed on to the mass analyzer part of the MS instrument. An important advantage of ESI is that it is a soft technique that allows e.g. non-volatile, thermolabile non covalent

biomacromolecular complexes to be ionized intact [544], offering the possibility of studying protein-drug complexes and other multimolecular complexes. Several kinds of mass analyzers with different properties are currently used in MS instrumentation, divided into two classes, beam analyzers and trapping analyzers. The beam analyzers are based on ions that leave the ionization source in a beam and pass through the analyzing field and on to the detector, and include mass analyzers called time-of-flight (TOF), sector analyzers, double focusing analyzers and quadrupole mass analyzers. The mass analyzers used in this thesis are the TOF [545] and quadrupole ion trap. The TOF mass analyser uses a simple technique where ions are separated based on their velocity. In theory the ions are all formed at the same time and are then allowed to accelerate through a fixed electric potential into a TOF drift tube that typically is 0.5-2 meters long. Each ion will receive the same kinetic energy in the acceleration process, and ions with a lower m/z ratio will move faster through the drift tube than ions with a high m/z ratio. The m/z of the ions is calculated from the time of ionization until the ions strike the detector. The quadrupole ion trap utilizes a three-dimensional electric field in order to trap ions inside the instrument. The ion trap is made up of two hyperbolic metal electrodes that face each other and that have a hyperbolic ring electrode halfway between each other. The ions are trapped in the space between the three electrodes by two types of electric fields; oscillating and non-static AC and non-oscillating and static DC fields. The ions are received into the trap by electrostatic gate pulses that open and close, allowing a certain number of ions to enter. An AC field is applied to the ring electrode in order to further focus the ions in the center of the trap. The trapped ions will oscillate with a certain frequency and are released from the trap if their trajectories become unstable. Making the trajectories unstable is achieved by changing the amplitude of the voltage so that the ions are sequentially released from low to high m/z . As the released ions collide with the detector into the detector mass spectrums are generated. Ions can also be ejected by inducing resonance conditions where the frequency of the ion is matched by a frequency of an additional potential applied to the end cap electrodes. The electric fields in the ion trap can also be used to induce fragmentation. Due to the focusing of and sequential release of ion “packages” the quadrupole ion trap has a very high level of sensitivity. The number of ions that can be allowed into the trap however is limited, as too many ions will reduce the overall performance of the trap due to electric field distortions.

NMR is a research technique that exploits the ability of magnetic atomic isotopes to (in a magnetic field) absorb and re-emit electromagnetic radiation in order to determine the physical, chemical and biological properties of single atoms or molecules. NMR is for instance utilized in medical imaging techniques of e.g. tissue (magnetic resonance imaging (MRI)), but in drug discovery NMR analysis provides information about the chemical structure, dynamics, reaction state and chemical environment of

molecules. NMR measurement is made possible due to atomic isotopes such as ^1H and ^{13}C that possess a magnetic moment due to a property called nuclear spin. Each nucleus in a molecule will be encompassed by a local magnetic field induced by the currents of electrons in the molecular orbitals and atoms surrounding it. The electron distribution around a nucleus depends on the binding partners, bond lengths and bond angles, resulting in differences in the local magnetic field around each nucleus. Hence, the NMR signal of an atom depends on the properties of its neighbors. This information can be used to determine the structure of the molecule. The magnetic properties correspond to specific resonance frequencies in a magnetic field, referred to as the chemical shift. The chemical shift of a certain nucleus in NMR spectroscopy is given as the resonant frequency relative to a standard. By measuring the chemical shifts in a sample molecule, it is possible to determine structural information about a molecule. For instance, the chemical shift of a proton neighboring an electronegative atom will be ~ 4 ppm, but closer to 0 when neighboring an electropositive group.

9. References

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10. Appendix II. Papers I-IV

A Novel Synthetic Smoothened Antagonist Transiently Inhibits Pancreatic Adenocarcinoma Xenografts in a Mouse Model

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Abstract

Background: Hedgehog (Hh) signaling is over-activated in several solid tumors where it plays a central role in cell growth, stroma recruitment and tumor progression. In the Hh signaling pathway, the Smoothened (SMO) receptor comprises a primary drug target with experimental small molecule SMO antagonists currently being evaluated in clinical trials.

Principal Findings: Using Shh-Light II (Shh-L2) and alkaline phosphatase (AP) based screening formats on a “focused diversity” library we identified a novel small molecule inhibitor of the Hh pathway, MS-0022 (2-bromo-N-(4-(8-methylimidazo[1,2-a]pyridin-2-yl)phenyl)benzamide). MS-0022 showed effective Hh signaling pathway inhibition at the level of SMO in the low nM range, and Hh pathway inhibition downstream of Suppressor of fused (SUFU) in the low μM range. MS-0022 reduced growth in the tumor cell lines PANC-1, SUIT-2, PC-3 and FEMX *in vitro*. MS-0022 treatment led to a transient delay of tumor growth that correlated with a reduction of stromal Gli1 levels in SUIT-2 xenografts *in vivo*.

Significance: We document the *in vitro* and *in vivo* efficacy and bioavailability of a novel small molecule SMO antagonist, MS-0022. Although MS-0022 primarily interferes with Hh signaling at the level of SMO, it also has a downstream inhibitory effect and leads to a stronger reduction of growth in several tumor cell lines when compared to related SMO antagonists.

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Competing Interests: The chemotype detailed in this study is part of a patent filed by Drs. Alex Khvat, Ilya Okun and others titled: HETEROCYCLIC INHIBITORS OF AN HH-SIGNAL CASCADE, MEDICINAL COMPOSITIONS BASED THEREON AND METHODS FOR TREATING DISEASES CAUSED BY THE ABERRANT ACTIVITY OF AN HH-SIGNAL SYSTEM (PCT/IB2008/055258; WO 2009/077956 A2 and WO 2009/077956 A3). There are currently no products arising from the work under development based on the chemotype detailed in this study. None of the authors, aside from Drs. Alex Khvat, Ilya Okun and Dan Holsworth who are employed by ChemDiv Inc, have any financial relationships that could be perceived as relevant. The authors declare no other competing interests. The competing interest detailed here does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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Introduction

The Hedgehog (Hh) signaling pathway is one of the key regulators in vertebrate development and is highly conserved among species from fruit flies to humans [1–4]. It is also one of the key pathways that regulate stem cells in the adult body [5]. Aberrant Hh signaling has been associated with a number of human tumors where the pathway has been implicated in tumor growth, malignancy, metastasis, and cancer stem cells [6–9]. Thus, the Hh pathway has become a focus for drug discovery and development [10–15].

The Hh pathway is unusual by several means, and central aspects of its functioning remain to be explored. The morphogens IHH, DHH and SHH interact with the 12-pass transmembrane receptor Patched (PTCH). PTCH inhibits the physically separate 7-pass transmembrane receptor Smoothened (SMO) by gating the

movement of SMO into cilia. Evidence suggests, that upon Hh binding, PTCH leaves the shaft of the primary cilium which allows SMO to enter from its inactive endosomal state into cilia [16–18]. Furthermore, it has been proposed that SMO exists in an inactive and active state [19,20] that may be regulated through a hypothesized sterol-like small molecule [4,19,21]. SMO migration into the primary cilium is followed by the inactivation of Suppressor of fused (SUFU) [22]. Current data suggest that SUFU, being a part of a multiprotein complex that also includes β-arrestin, KIF3a and IFT88, impedes the nuclear localization of GLI proteins [16,17,22]. In addition it may act as a nuclear co-repressor [23]. SUFU is ubiquitinated upon the activation of Hh signaling which initiates its degradation in the proteasomes [24] leading to the release of GLI2/3 into the nucleus where they regulate transcription of downstream target genes including the activating transcription factor GLI1. Although GLI1 presence in

the nucleus is primarily a consequence of active Hh signaling, it can be attenuated by other signaling pathways [25].

There are several key mechanisms in tumorigenesis that may involve Hh/GLI signaling [11,13]; first, inactivating mutations in the negative regulators PTCH or SUFU, or activating mutations in the positive regulator SMO cause pathway activation in a cell-autonomous and Hh ligand independent manner [5,26–28]; secondly, ligand-dependent autocrine mechanisms in which cancer cells both secrete and respond to Hh ligands causing cell-autonomous pathway activation [29,30]; thirdly, paracrine mechanisms in which stromal cells are induced by Hh producing cancer cells [31–34]. Both autocrine and paracrine effects can lead to heterogeneity with respect to Hh pathway activity within a tumor [35]. Several SMO antagonists have been developed and early data show clinical efficacy in selected tumors [36]. However, there has been some debate whether the *in vivo* growth inhibition observed for Hh antagonists is due to inhibition of autocrine or paracrine Hh signaling. Several recent studies suggest that the primary role of Hh inhibition in Hh secreting tumors may be due to the inhibition of paracrine signaling involving tumor-stroma interactions [33,37–41]. In particular, tumor derived SHH has been shown to promote desmoplasia in pancreatic cancer [42], where the induced stroma in combination with poor vascularization may act as a barrier that is linked to a poor response to chemotherapy [40,41].

Following the identification of cyclopamine as a natural SMO inhibitor [43–45], several Hh pathway antagonists have been reported that either act at the level of SMO [46], GLI1 [47], or other parts of the pathway [10,13,36]. Among these inhibitors, some have been progressed to clinical trials. One of these, GDC-0449 [15,34,48], is currently in several phase I and phase II clinical trials for various types of cancers, including pancreatic cancer (trial ID: NCT01064622 and NCT00878163). Also, the cyclopamine derivative IPI-926 [14] has been through a phase I clinical trial in patients with non-disclosed advanced and/or metastatic solid tumors, and is currently in a phase Ib/II clinical trial in patients with untreated metastatic pancreatic cancer (trial ID: NCT01130142).

Here, we describe the identification and evaluation of a novel small molecule SMO antagonist, MS-0022. MS-0022 displays a differential efficacy on various solid tumors *in vitro* and on PANC-1 and SUIT-2 xenografts *in vivo*. The reported findings are a further confirmation of the potential of small molecule Hh antagonists as anticancer agents.

Results

Identification of the novel Hh antagonist MS-0022

To identify novel antagonists to Hh signaling, a focused diversity library of 12,000 compounds (10 μ M) was screened using C3H10T1/2 cells induced by recombinant human SHH and employing an alkaline phosphatase (AP) readout in high throughput format [49], followed by a verification step using Shh-L2 cells. MS-0022 (2-bromo-*N*-(4-(8-methylimidazo[1,2-*a*]pyridin-2-yl)phenyl)-benzamide), was identified as a potent Hh pathway antagonist with an IC₅₀ of 100 nM in Shh-L2 cells. The structure of MS-0022 was confirmed by ¹H and ¹³C NMR (Table S1 and Figure S1). In order to explore the parts of the core structure required for activity in MS-0022, a small scale broad structural analysis was performed based on activity inhibition in Shh-L2 cells. As shown in Table 1, a deletion of the 2-bromophenyl or the Imidazo[1,2-*a*]pyridine moiety of MS-0022 led to a substantial loss of activity (for the structure backbone see Figure 1A). The activity was partially retained when replacing the Imidazo[1,2-*a*]pyridine system with a naphthalene-2-ylcarbamoyl system (MS-0018) or a 6-morpholinopyridazin-3-yl system (MS-0015). A further focused structural analysis of a limited number of MS-0022 analogs is shown in Table 2 (for the base structure see Figure 1B).

Changing R2 from a hydrogen to a methyl group reduced activity 6-fold (MS-0030). Incorporation of a nitrogen atom in position Y reduced activity 23-fold (MS-0031). Substitution of R3 with a 2-fluorophenyl group reduced activity 1.6-fold (MS-0032). Interestingly, if R1 was replaced with a hydrogen and R3 with 4-methoxyphenyl, the activity dropped 1.8-fold (MS-0033). If R1 of MS-0033 was replaced with a nitrogen atom in the ring (MS-0035), the activity dropped an additional 8-fold. In general, a nitrogen atom in the 8-position of the imidazo[1,2-*a*]pyridine ring system caused a significant reduction in activity. Furthermore, a nitrogen atom placed in position Y impacted the activity negatively.

A dose response curve of MS-0022 in Shh activated Shh-L2 cells, is shown in Figure 1C, using cyclopamine and GDC-0449 as a comparison. MS-0022 exhibited an IC₅₀ of 100 nM, while cyclopamine exhibited an IC₅₀ of 210 nM and GDC-0449 an IC₅₀ of 30 nM.

To ascertain that the compounds interacted at the level of SMO, the most potent compounds, MS-0022, MS-0032 and MS-0033 were shown to compete with BODIPY-cyclopamine with IC₅₀'s of 259, 93 and 287 nM respectively (Table 2). Thus, MS-0022 and analogs of MS-0022, as well as GDC-0449, inhibit Hh

Table 1. Activity of MS-0022 and deletion/substitution analogs (See structure in figure 1A).

ID	R1	R2	R3	MW	IC50 (Shh-L2, nM)
MS-0022	8-methylimidazo[1,2- <i>a</i>]pyridine	–	2-bromophenyl	406.3	100
MS-0011	8-methylimidazo[1,2- <i>a</i>]pyridine	–	Methyl	265.3	>20,000
MS-0012	8-methylimidazo[1,2- <i>a</i>]pyridine	–	2-propyl	293.4	>20,000
MS-0013	–	O-Amide	2-bromophenyl	319.2	>20,000
MS-0014	Propyl	–	2-bromophenyl	318.2	10,000
MS-0015	6-morpholinopyridazin-3-yl	–	2-bromophenyl	439.3	800
MS-0016	(6-phenylpyrimidin-4-yl)amino	–	2-bromophenyl	445.3	2000
MS-0017	naphthalen-2-ylcarbamoyl	–	2-bromophenyl	445.3	280
MS-0018	2-benzylidenehydrazinacarbonyl	–	2-bromophenyl	442.3	5000

Table depicting molecular weight and biological activity (nM IC₅₀) of deletion or substitution analogs of MS-0022 as measured by pathway inhibition in Shh induced Shh-L2 cells.

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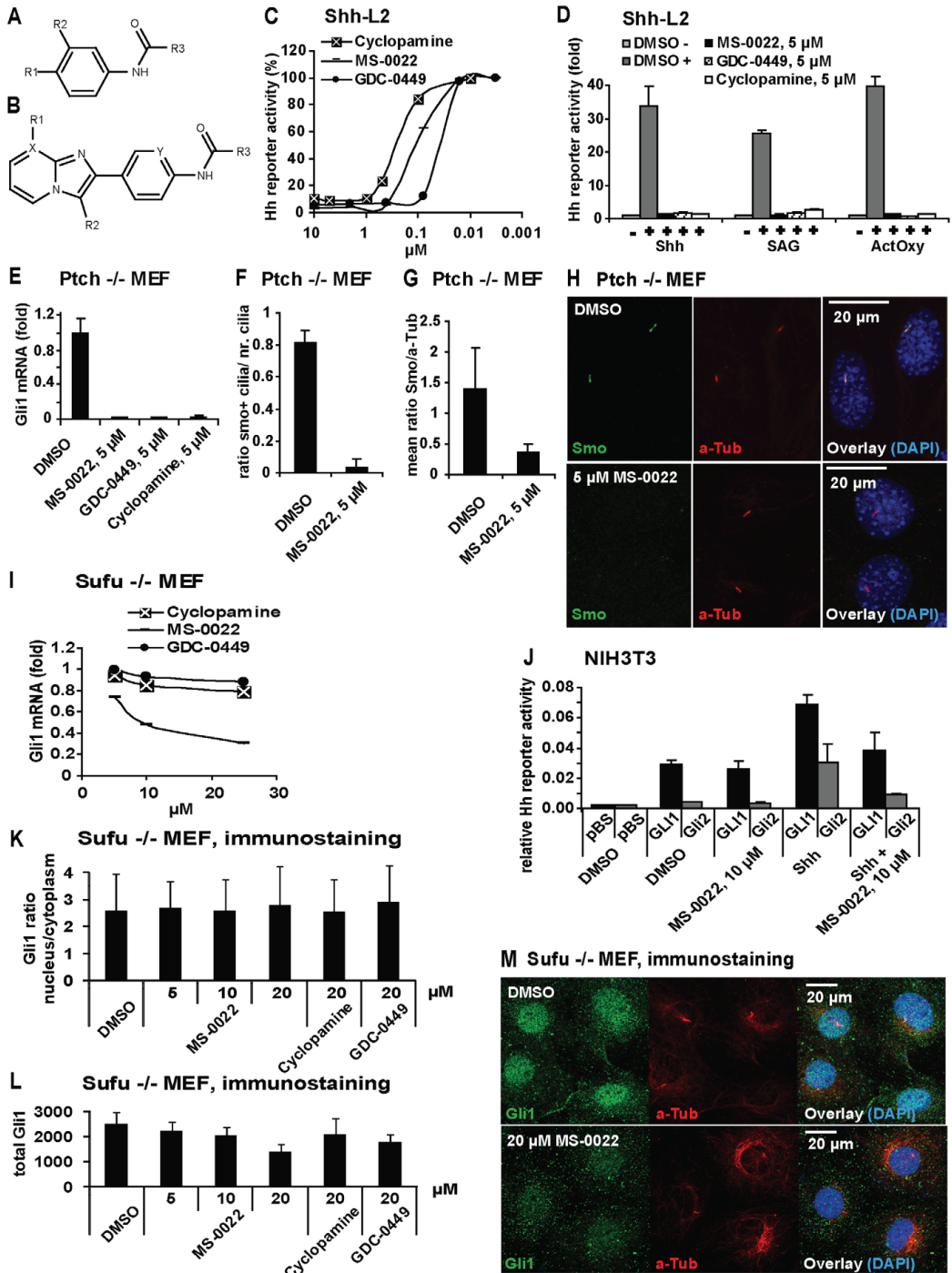


Figure 1. Identification and activity of MS-0022. A) Structure for Table 1. B) Structure for Table 2. C) Dose response curves of indicated compounds in Shh activated Shh-L2 cells after 48 hours treatment (n=8). D) Shh-L2 cells activated with Shh, 200 nM SAG and ActOxs (5 μ M+5 μ M) treated with indicated compounds at 5 μ M for 48 hours. Graph displays fold Hh reporter activity with standard deviation (SD) (n=3). The basal activity (DMSO) was set as = 1. E) Normalized (GAPDH) Gli1 mRNA expression in Ptch^{-/-} MEF after 48 hours of treatment with indicated compounds at 5 μ M. Graph displays fold expression compared to DMSO control, with SD (n=3). F–H) Immunostaining of Ptch^{-/-} MEF cells treated with MS-0022 or DMSO control for 24 hours (anti-smoothed (Smo) in green, anti acetylated Tubulin (aTub) in red, DAPI in blue). Treated and control culture slides (n=3) were stained and 3 regions on each slide was imaged and analyzed. F) Graph displaying the ratio of Smo positive cilia versus total number of cilia in stained cells by eye count, with SD. G) Graph displaying the ratio of mean ciliary intensity of Smo staining versus aTub as quantified using ImageJ software, with SD. I) Dose response curve of MS-0022, GDC-0449 and cyclopamine by normalized (GAPDH) Gli1 mRNA expression in Sufu^{-/-} MEF after 72 hours treatment (n=3). J) NIH3T3 cells cotransfected with GliB5-Luc in combination with pBluescript (pBS), FL-Gli1-HA or Gli2-GFP, were treated with DMSO, 50% Shh conditioned medium, 10 μ M MS-0022 and a combination of Shh and 10 μ M MS-0022 for 48 hours. Graph displays relative Hh pathway activity (reporter firefly luciferase normalized for renilla transfection control), with SD (n=3). K–M) Immunostaining of Sufu^{-/-} MEF cells treated with DMSO control, MS-0022 (5, 10 and 20 μ M), cyclopamine (20 μ M) and GDC-0449 (20 μ M) for 48 hours (n=3). K) Graph displaying ratio of nuclear Gli1 staining intensity versus cytoplasmic Gli1 staining intensity, with SD. L) Graph displaying sum of nuclear and cytoplasmic Gli1 staining intensity in treated cells, with SD.

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signaling at the level of SMO. MS-0022 inhibited SMO with similar affinity as cyclopamine.

MS-0022 blocks Hh signaling at the level of SMO, but has additional activity downstream of SUFU

MS-0022 was shown to compete with BODIPY-cyclopamine, and the observed effect on C3H10T1/2 cells and Shh-L2 cells suggested that MS-0022 blocks Hh signaling at the level of SMO. However, to further verify the function of MS-0022 as a SMO blocker, the activity of MS-0022 was evaluated in experiments that explore the various modes of Hh signaling induction in mouse cell lines. Upstream of Gli, Hh signaling can be induced by the Hh morphogens; by functional loss of Ptch, as present in the constitutively active Ptch^{-/-} mouse embryonic fibroblast (MEF) cell line; by a combination of the activating oxysterols hydroxycholesterol 20- α and hydroxycholesterol 22S (ActOXS) and by the SMO agonist SAG and by Sufu inactivation, as present in Sufu^{-/-} MEFs. As seen in Figure 1D, MS-0022 blocked Hh pathway induction in Shh, SAG and ActOXS induced Shh-L2 cells similarly to GDC-0449 and cyclopamine. MS-0022 also reduced Gli1 mRNA expression in Ptch^{-/-} MEFs, similar to GDC-0449 and cyclopamine (Figure 1E). Cyclopamine blocks Smo function within primary cilia without blocking its translocation to the cilia. In contrast, MS-0022 effectively blocked ciliary accumulation of Smo (Figure 1F–H), indicating an effect on the level of Smo similar to GDC-0449, but divergent from

cyclopamine. Primary cilia in reporter cells were identified using an antibody against the ciliary maker acetylated tubulin. Acetylation of tubulin is found in stabilized microtubular structures like cilia and mitotic spindles, and has effectively been used as a marker for cilia in various studies [50,51].

As expected for a SMO antagonist, cyclopamine and GDC-0449 exhibited little inhibitory effect on Hh signaling downstream of Smo in Sufu^{-/-} MEFs. In contrast, MS-0022 reduced relative Gli1 mRNA levels Sufu^{-/-} MEFs by 50% at a dose of 10 μ M (Figure 1I). Further downstream, at the level of Gli1 or Gli2, Hh pathway inhibitory effect by MS-0022 was not detected as indicated by the lack of inhibition of a forced expression of either of the two transcription factors with 10 μ M of MS-0022 (Figure 1J). MS-0022 was able to show partial inhibition (Figure 1J) of Gli1 or Gli2, only when the Hh signaling pathway was further activated by Shh. In order to further elucidate the activity of MS-0022, Sufu^{-/-} MEF cells that have been treated with MS-0022, cyclopamine or GDC-0449 were stained with an antibody recognizing Gli1. The intensity of Gli1 staining in the nucleus and the cytoplasm was measured, and neither of the treatments resulted in a shift in the ratio of Gli1 in the nucleus versus the cytoplasm (Figure 1K). Thus the reduction of Gli1 mRNA levels in the Sufu^{-/-} MEF cells by MS-0022 was not a consequence of Gli1 relocation in the cell. However, while the treatment with 20 μ M MS-0022 did not alter the ratio of nuclear versus cytoplasmic Gli1, the total level of Gli1 in the cells was reduced (Figure 1L–M), correlating well with the

Table 2. Activity analysis of MS-0022 chemotype (See structure in figure 1B).

ID	R1	R2	R3	X	Y	MW	IC50 (Shh-L2, nM)	IC50 (nM, BODIPY-cyclopamine inhib.)	cLogP	PSA
MS-0022	CH3	H	2-bromophenyl	C	C	406.3	100	259	5.44	46.4
MS-0030	CH3	CH3	2-bromophenyl	C	C	420.3	630	–	5.86	46.4
MS-0031	CH3	H	2-bromophenyl	C	N	407.3	2300	–	3.88	59.29
MS-0032	CH3	H	2-fluorophenyl	C	C	345.4	161	93	4.39	46.2
MS-0033	H	H	4-methoxyphenyl	C	C	343.4	181	287	4.24	55.63
MS-0034	–	H	2-methoxyphenyl	N	C	344.4	>10,000	–	3.21	68.5
MS-0035	–	H	4-methoxyphenyl	N	C	344.4	877	–	3.1	68.52
MS-0036	–	H	3,5-dimethylphenyl	N	C	342.4	3,700	–	4.06	59.29
MS-0037	–	H	3-chlorophenyl	N	C	348.8	1,870	–	3.87	59.29
MS-0038	–	H	2-trifluoromethylphenyl	N	C	382.3	1,150	–	2.98	59.3

Biological activity of side group analogues of MS-0022 measured by pathway inhibition in Shh induced Shh-L2 cells and by BODIPY-cyclopamine competition, including cLogP (computed LogP, octanol/water partition coefficient) and polar surface area (PSA).
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reduction in Gli1 mRNA. At the same dose GDC-0449 also reduced Gli1 levels in the cells, but to a lesser extent than MS-0022 (Figure 1L), while cyclopamine had no significant effect.

We conclude that MS-0022 acts at the level of SMO blocking its ciliary transport in the nanomolar range. An additional inhibitory effect of MS-0022 on Hh signaling downstream of SuFu that required a dose in the 10–20 micro molar range is linked to a reduction of Gli1 protein levels.

MS-0022 blocks tumor growth in pancreatic adenocarcinoma, prostate carcinoma and melanoma cell lines *in vitro*

To test *in vitro* efficacy of MS-0022, we profiled the presence of central components of the Hh signaling pathway in the pancreas adenocarcinoma cell lines PANC-1 and SUT-2, the prostate cancer cell line PC-3, and the melanoma cell line FEMX by real time PCR (Table 3). Although all cell lines expressed detectable levels of GLI1 mRNA, the level of expression varied, as did other components of the Hh signaling pathway. However, the clear presence of the direct Hh downstream marker PTCH1 in all cell lines, indicated Hh/GLI1 pathway activity.

To determine if GLI1 mRNA levels in the tumor cells could be reduced by MS-0022, real time PCR was carried out on cells treated with different doses of MS-0022, cyclopamine and GDC-0449 for 48 hrs (Figure 2A). In parallel, growth inhibition was measured by MTS (Figure 2B). For the PANC-1 cell line, growth inhibition and reduction of GLI1 mRNA levels upon treatment, correlated well at 10 μ M for all three compounds. At 5 μ M, however, MS-0022 and GDC-0449 reduced growth without reducing GLI1 mRNA levels. In the SUT-2 cell line, MS-0022, cyclopamine and GDC-0449 all reduced GLI1 mRNA levels, but only MS-0022 reduced growth. For the PC-3 cell line, both MS-0022 and GDC-0449 reduced GLI1 mRNA levels, although growth was only reduced by MS-0022. For the FEMX cell line, the growth and GLI1 mRNA levels correlated well at 10 μ M, but not at 5 μ M. In conclusion, using the SMO antagonists GDC-0449 and cyclopamine, no correlation between growth inhibition and reduction of GLI1 mRNA levels could be detected in the four tumor cell lines PANC-1, SUT-2, PC-3 and FEMX. However, a correlation between growth inhibition and GLI1 mRNA levels were apparent at a dose of 10 μ M MS-0022 across all four tumor cell lines. The data set is consistent with an additional Hh pathway inhibitory effect of MS-0022 downstream of SMO/SUFU that requires relative higher doses of the compound as compared to direct SMO inhibition. As seen in Figure 2B, both CDG-0449 and cyclopamine did not lead to more than 30% growth reduction in the tested cell lines PANC-1, SUT-2, PC-3 and FEMX during a 4 day exposure to 10 μ M compound in a MTS assay. In contrast, at the same dose, MS-0022 reduced growth from 40%–70% in the same cell lines (Figure 2B). An immortalized,

non-tumorigenic hepatocyte cell line, THLE-2, was included as a control, and the THLE-2 cells responded with a 25%–30% growth reduction to 10 μ M compound exposure possibly indicating a weak Hh dependency in this control cell line (Figure 2B).

To address growth inhibition in a model relevant to xenograft studies, PANC-1 and SUT-2 cells were seeded in a soft agar colony forming assay. A dose response curve was generated for MS-0022, while using GDC-0229 and cyclopamine as controls. For both cell lines, treatment with MS-0022 led to a reduction in large (Figure 2C–D, and G) and medium sized colonies in a dose dependant manner (Figure 2E–F, and G). Also upon MS-0022 treatment, an increase in the number of small colonies was observed, indicating that the reduced growth of the small and medium sized colonies is linked to decreased proliferation rather than apoptosis. The cyclopamine control was excluded from the dataset due to problems with crystallization of cyclopamine in the soft agar.

Long term growth assays with 2–3 serial passages (5–7 days per passage) further confirmed the efficacy of MS-0022 on the tested PANC-1, SUT-2, PC-3 and control THLE-2 cell lines (Figure 2H). In the presence of 5 μ M MS-0022, there was an initial growth reduction in the first passage of the control THLE-2 cells, but by the second passage the growth was not affected by the treatment. In contrast, the growth was reduced by 80% in PANC-1 cells and PC-3 cells after passage 2. Serial passage growth reduction was not significant for passage 2 and 3 in the SUT-2 cells.

Pathway specificity

To address possible effects of MS-0022 on other central signaling pathways, we analyzed whether MS-0022 affected Wnt and TNF- α signaling using firefly luciferase reporter assays. As seen in Figure 3A, MS-0022 did not significantly block L1 medium induced Wnt signaling in HEK293 cells, nor did it block TNF- α induced Nf κ B signaling in NIH3T3 cells at 10 μ M and 20 μ M (Figure 3B). Instead, MS-0022 led to a slight increase of Nf κ B signaling (Figure 3B). In addition, MS-0022 was tested at 10 μ M in a Millipore diversity panel comprised of 58 kinases. MS-0022 did not significantly inhibit the activity of any of the tested kinases (Table 4).

Efficacy and bioavailability of MS-0022 in an *in vivo* pancreatic adenocarcinoma xenograft model

In preparation for *in vivo* xenografts, the bioavailability of MS-0022 in mouse plasma was evaluated. The maximum concentration (C_{max}) of the compound in plasma was 934 ng/mL upon a 5 mg/kg IV injection. For a 5 mg/kg IP injection, it was 378 ng/mL and after 5 mg/kg PO administration, it was 912 ng/mL (Figure 3D–F and Table 5). Per oral bioavailability was calculated to be 98%. The half-life (T_{1/2}) after MS-0022 administration was between 55 and 60 minutes, indicating that MS-0022 has a moderate stability in plasma. Compound concentration in mouse liver tissue 2 hours after IP administration was 244 ng/g. Thus, MS-0022 readily enters tissue from the plasma. Overall, the compound exhibited good exposure and moderate stability in plasma.

Next the *in vivo* tolerability of MS-0022 was examined. No statistically significant alterations in body weight were observed in animals treated with daily IP injections of 200 mg/kg of MS-0022 for 5 days (Figure 3G), indicating that the compound was well tolerated. Mice did not show any outward signs of toxicity or other side effects (weight loss, fur ruffling, hunched posture). Even though there were no outward signs of side effects or toxicity, we analyzed the AST/ALT levels as a measure of liver toxicity in xenografted animals that had been treated with MS-0022 for a

Table 3. Expression profile, Tumor cell lines.

	PTCH1	SMO	GLI1	GLI2	GLI3	SHH	HIP	SUFU
PANC-1	+++	+	+++	+++	+	+++	+/-	+++
SUT-2	++	++	++	++	nt	+++	nt	nt
PC-3	++	++	++	+	+	+++	+	+
FEMX	++	+	+	+	-	-	-	+

Expression analysis of tumor cell lines according to the PCR cycle where amplification was detected, starting with 1 μ g mRNA (+++ = 20–25, ++ = 25–30, + = 30–35, - = >35 and nt = not tested).

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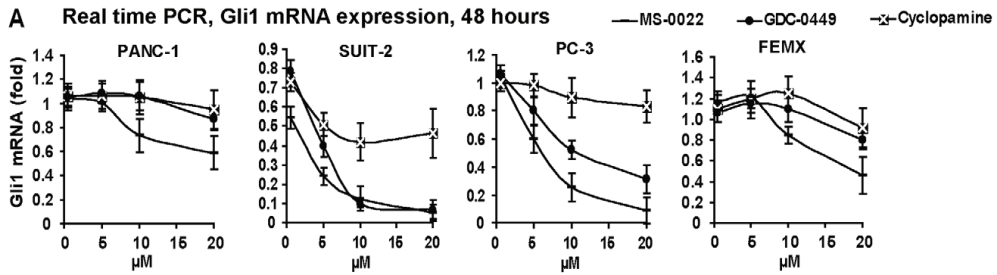
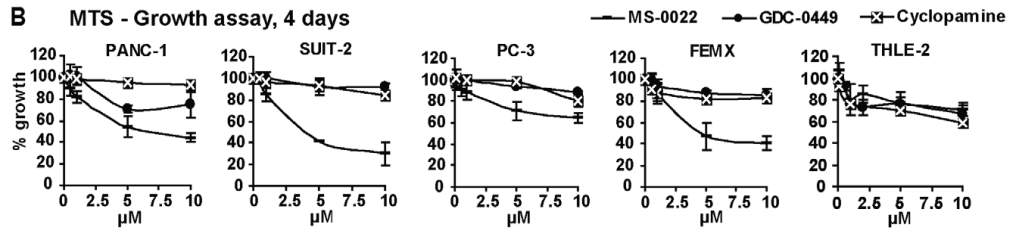
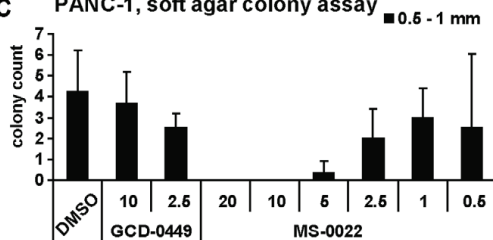
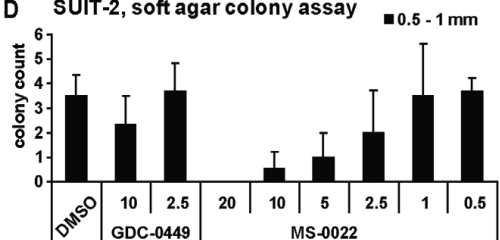
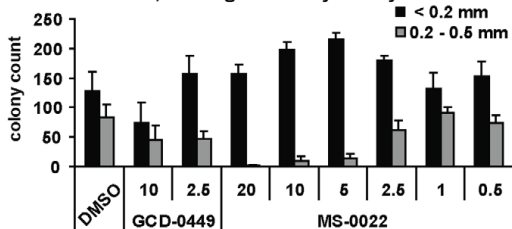
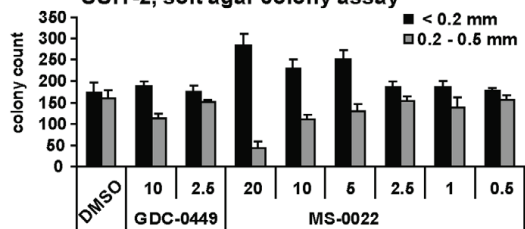
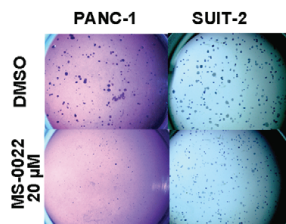
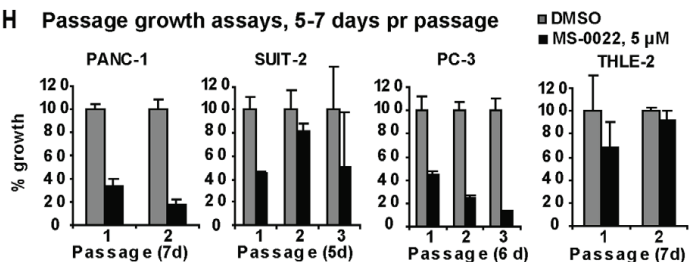
A Real time PCR, Gli1 mRNA expression, 48 hours**B MTS - Growth assay, 4 days****C PANC-1, soft agar colony assay****D SUIT-2, soft agar colony assay****E PANC-1, soft agar colony assay****F SUIT-2, soft agar colony assay****G Soft agar colony assay****H Passage growth assays, 5-7 days pr passage**

Figure 2. *In vitro* activity in tumor cell lines and a control cell line. A) Graphs displaying dose response curves of normalized (GAPDH) Gli1 mRNA expression in indicated tumor cell lines after 48 hour treatment of MS-0022, cyclopamine and GDC-0449. Graphs include standard deviation (n=3). B) Graphs displaying 4 day dose response curves of cell growth as a % growth of DMSO control (MTS) for PANC-1, SUIT-2, PC-3, FEMX and THLE-2 cells treated with MS-0022, GDC-0449 and cyclopamine at the indicated dose, with SD (n=8) C-G) Soft agar colony formation assay of PANC-1 (21 days) and SUIT-2 (14 days) cells treated with DMSO or MS-0022 and GDC-0449 at the indicated doses (n=3). Each well was imaged and colonies were counted by eye according to size. Graphs displaying the colony count of larger colonies (0.5–1 mm) are shown in C) for PANC-1 and D) for SUIT-2, with SD. Graphs displaying the colony count of medium (0.2–0.5 mm) and small (<0.2 mm) colonies are shown in E) for PANC-1 and F) for SUIT-2, with SD. H) Passage growth assays with PANC-1, SUIT-2, PC-3 and THLE-2 cell lines treated with DMSO control or 5 μ M MS-0022 for 2–3 passages of 5–7 days, displayed as % growth of DMSO control, with SD (n=3). doi:10.1371/journal.pone.0019904.g002

longer time period than 5 days. As the liver normally acts as the primary site of drug metabolism, AST/ALT serum levels is an easily obtainable measure of toxic side effects. As seen in Figure 3C, AST/ALT levels remained greater than 1 in mice treated with 50 mg/kg of MS-0022 after a 30 days injection scheme (5 day injection, 2 day pause), suggesting that at this dose MS-0022 was well tolerated without apparent toxic effects in the liver in comparison to the control treated mice.

In vivo efficacy of MS-0022 was tested against two pancreatic adenocarcinoma cell lines PANC-1 and SUIT-2. For PANC-1, subcutaneous tumors were established in CB17/SCID mice (n=6). Mice were randomized and dosed with control solvent (water with 1% Tween 80) or MS-0022 (QD, IP at 50 mg/kg) in a 5 day injection/2 day pause scheme. Tumor volumes were evaluated throughout the treatment by measuring two perpendicular diameters with calipers, and by calculating tumor volume (mm^3) using the formula $V = a \times b^2 \times \pi / 6$. At the end of the treatment there was a 38% reduction in tumor volume and a 33% reduction in tumor weight compared to tumors in control solvent treated littermates (Figure 3H–I). The growth curve (Figure 3H), shows that the tumors in both control and treated groups eventually reached a similar growth pattern, and the growth inhibition by MS-0022 appeared to be transient. At the endpoint of the experiment, no statistically relevant difference in human (tumor) or mouse (stroma) Pth1 or Gli1 mRNA levels could be identified in the extracted tumor mRNA ($P>0.05$) (Figure 3J).

Compared to PANC-1, SUIT-2 cells displayed a stronger responsiveness to MS-0022 *in vitro*, both in the reduction of Gli1 expression and in growth (MTS assay). Therefore, the effect of MS-0022 was also tested *in vivo* using SUIT-2 pancreas adenocarcinoma cells, and subcutaneous tumors were established in CB17/SCID mice (n=11). Mice were randomized and dosed daily with solvent or 50 mg/kg MS-0022 via PO administration. The MS-0022 treatment did not result in any outwards signs of toxicity in the mice, and animal weight (Figure 4C) was unaffected by the treatment when compared to the animals that received control solvent. After 7 days, 3 mice from each treatment group were sacrificed and the tumors were harvested for analysis, while the remaining animals (n=8) were sacrificed after 18 days of treatment, due to tumor burden. In general, the SUIT-2 tumors showed a more aggressive growth in comparison to the PANC-1 tumors. At the endpoint of the experiment, a 27% reduction in tumor volume and a 36% reduction in tumor weight were measured in treated animals compared to the control group (Figure 4A–B). Similar to PANC-1 xenografts (Figure 3D), a transient delay of growth was observed during the first days of treatment, followed by a recovery of growth with growth rates similar to untreated tumors. As in PANC-1 xenografts, no statistical relevant reduction of human or mouse Gli1, or Pth1 mRNA levels was detectable at the endpoint of the experiment (Figure 4E). However, when samples from the SUIT-2 xenografts were analyzed after 7 days of treatment only, mouse (stromal) Gli1 mRNA in the tumors was reduced

significantly ($P<0.001$), while both human and mouse PTCH and human Gli1 remained unaltered ($P>0.05$) (Figure 4D). There was no detectable difference in animal weight between the control and MS-0022 treated animals (Figure 4C). As inhibition of SMO has been linked to an increase in vascularization in poorly vascularized tumors [41], we investigated the vascularization of the tumor tissue upon MS-0022 treatment. Samples from the harvested tumors were cryosectioned, fixed and stained for the presence of the endothelial cell marker (CD31), as a marker for microvessels in tumor tissue. Large variations in SUIT-2 tumor shape including the occurrence of necrotic cavities, led to substantial variations in the vascularization of the tumors in all samples derived after 7 or 18 days of treatment and in control samples. Due to substantial variability, no apparent difference between the vascularization of samples derived from treated and untreated animals was detectable. Irrespective of MS-0022 treatment, CD31 staining revealed high vascularization at the tumor edge, while vascularization in the center of the tumor was generally low (Figure 4F).

Discussion

MS-0022 was identified as a potent antagonist of Hh signaling that blocks the translocation of SMO to the cilia displaying a transient *in vivo* antagonistic effect in a pancreatic adenocarcinoma xenograft model.

MS-0022 contains a core structural motif common to other SMO antagonists. Analysis of the core structure of MS-0022 revealed that the “phenyl-amide-phenyl” (Figure 1A, where R3 = phenyl) portion of the molecule was also present in HhAntag [33], GDC-0449 [52], Sant-2 [53] and Compound Z [54]. Deleting the 2-bromobenzene moiety of MS-0022 resulted in a total loss of activity, confirming the importance of the identified core structure for the activity of this class of molecules. Interestingly, although the “phenyl-amide-phenyl” core is identical in all of these molecules, there are also important differences in the structures. HhAntag, Sant-2 and Compound Z are closer to each other in structure than MS-0022 and GDC-0449, as they share a 1H-benzimidazo structure coupled in the 3 position to the 4-chloro-N-phenyl. Interestingly, when the imidazo[1,2-a]pyridine of MS-0022 was deleted, activity was reduced. However, when it was substituted with groups that contain nitrogen in ortho position to the N-phenyl, partial activity was retained. Similar to GDC-0449 and cyclopamine, MS-0022 inhibited the Hh pathway activity after induction by various agonistic factors like Shh, oxysterols and SAG. The ability of MS-0022 to compete with BOPIDY-cyclopamine binding suggests that it acts by a similar mode of action as structurally related SMO antagonists, such as GDC-0449. This was further confirmed by the inhibition of SMO translocation to the primary cilium by MS-0022. While MS-0022 inhibited SMO in the nanomolar range, it displayed an additional effect on the Hh pathway downstream of SMO that required a higher dose. In the micromolar range, MS-0022 led to a significant

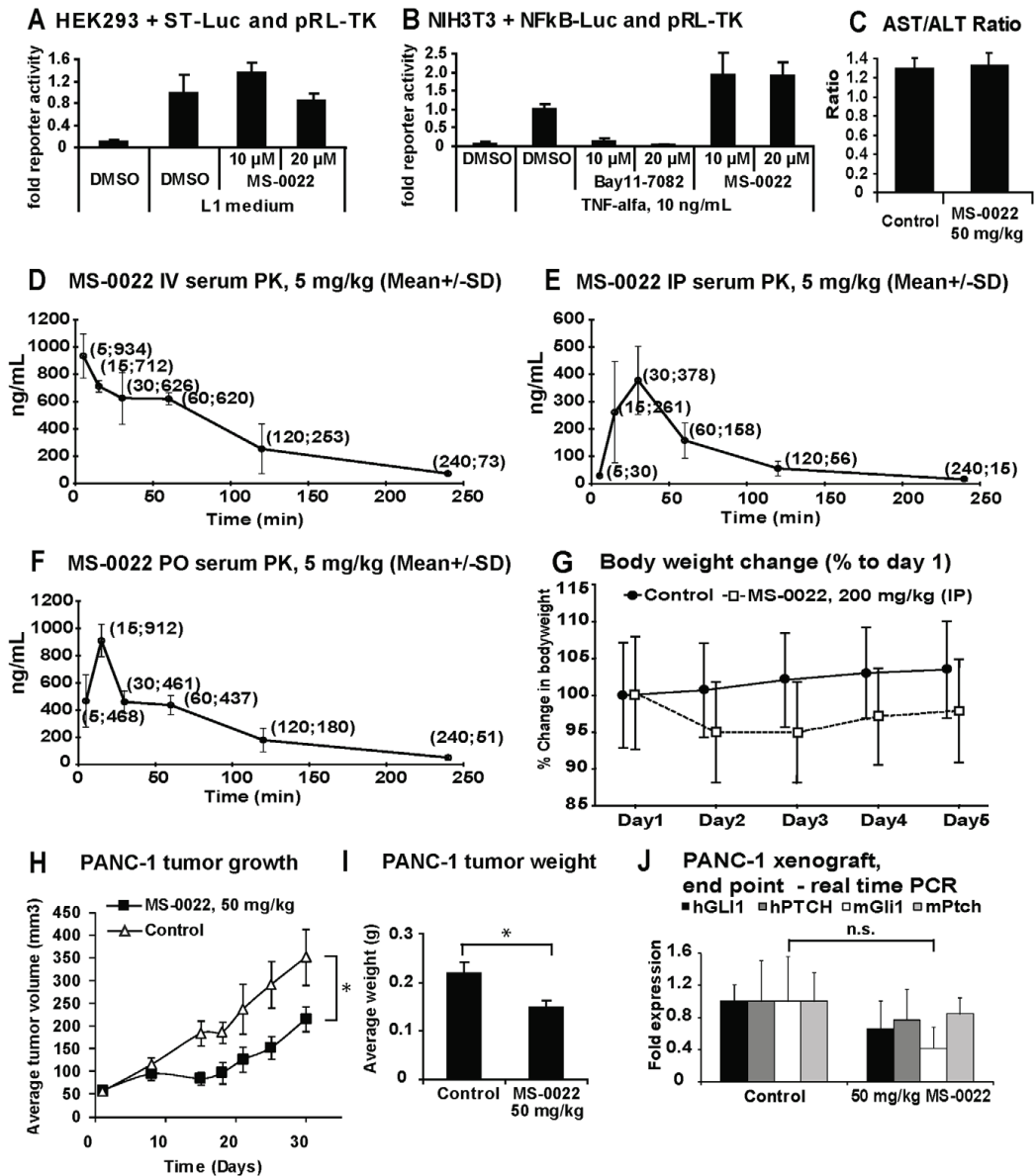


Figure 3. In vitro MS-0022 pathway selectivity, in vivo tolerance and distribution, and activity in a PANC-1 xenograft model. A) HEK293 cells cotransfected with SuperTop-luc (ST-Luc) and pRL-TK, were treated with control medium and L1 medium \pm 10 and 20 μ M MS-0022 for 24 hours. Graph displays fold reporter activity with standard deviation ($n=3$). B) NIH3T3 cells cotransfected with NFkB-luc and pRL-TK, treated with control medium and 10 ng/mL TNF- α \pm 10 and 20 μ M NFkB inhibitor Bay11-7082 or 10 and 20 μ M MS-0022 for 24 hours. Graph displays fold reporter activity with SD ($n=3$). C) Graph depicting average AST/ALT ratio from mouse blood at end of animal treatment in the PANC-1 xenograft. D-F) Blood distribution curves after IV, IP and PO administration of 5 mg/kg MS-0022 in mice. Graphs display mean dose (ng/mL by time) \pm SD. G) Body weight change in animals treated with 200 mg/kg MS-0022 over 5 days, with SD ($n=5$). H-J) Analysis of subcutaneous PANC-1 tumors in mice treated with solvent control or 50 mg/kg MS-0022 for 21 days ($n=6$) by IP administration. H) Growth curves of PANC-1 tumors in mice after 30 days of treatment, showing average tumor volume (mm³) with standard error of the mean (SEM). I) Graph displaying average PANC-1 tumor weight at end of treatment, with SD. J) Graph displays normalized (GUSB) fold expression of mouse and human Gli1 and Ptch in treated tumors, with SD ($n=3$). * = P value <0.05 . (n.s. = P value >0.05). doi:10.1371/journal.pone.0019904.g003

Table 4. Kinase activity in presence of 10 μ M MS-0022 in a kinaseprofiler diversity array.

kinase:	Activity %	kinase:	Activity %	kinase:	Activity %
Abl(h)	96	EphB4(h)	99	NEK2(h)	127
ALK(h)	95	Fyn(h)	100	p70S6K(h)	94
AMPK(r)	98	GRK5(h)	119	PAK2(h)	93
ASK1(h)	91	GSK3 β (h)	88	PDGFR β (h)	108
CaMKI(h)	106	IGF-1R(h)	105	Pim-1(h)	103
CDK1/cyclinB(h)	110	IKK α (h)	103	PKA(h)	100
CDK2/cyclinA(h)	98	IRAK4(h)	95	PKB α (h)	107
CDK7/cyclinH/MAT1(h)	103	JAK2(h)	110	PKC α (h)	106
CDK9/cyclin T1(h)	98	JNK3(h)	71	PKC θ (h)	103
CHK1(h)	84	KDR(h)	103	PKG1 α (h)	94
CK1 γ 1(h)	93	LOK(h)	93	Plk3(h)	107
CK2 α 2(h)	109	Lyn(h)	103	PRAK(h)	71
cKit(h)	109	MAPK2(h)	101	ROCK-I(h)	103
c-RAF(h)	106	MAPKAP-K2(h)	92	Rse(h)	107
cSRC(h)	83	MEK1(h)	73	Rsk1(h)	91
DRAK1(h)	97	MKK7 β (h)	109	SAPK2a(h)	102
DYRK2(h)	97	MLK1(h)	103	SRPK1(h)	102
eEF-2K(h)	99	Mnk2(h)	90	TAK1(h)	98
EGFR(h)	84	MSK2(h)	92		
EphA5(h)	112	MST1(h)	94		

Millipore diversity panel - percent activity of tested kinases in the presence of 10 μ M MS-0022.
doi:10.1371/journal.pone.0019904.t004

reduction of Gli1 mRNA and protein levels in Sufu $-/-$ cells. This effect was not due to an altered or inhibited translocation of Gli1 to the nucleus, indicating that MS-0022 did not reduce Gli1 levels by direct inhibition of Gli1. The precise cause of this secondary effect remains to be elucidated.

As the implications of aberrant Hh signaling are getting increasingly evident in several cancers, pathway antagonists will be an essential toolkit for development of future treatments. The emerging data from clinical trials with GDC-0449 show both the benefits and possible pitfalls of a pure SMO antagonist; a clear tumor response in Hh driven tumors such as basal cell carcinoma and medulloblastoma [34,55,56], and the occurrence of a drug induced resistance caused by mutations in the Smo locus [34,55].

Table 5. Pharmacokinetics of MS-0022.

	Cmax, ng/ml	AUClast ng*min/ml	AUCinf ng*min/ml	T1/2, min	Kel, min-1
IV	934.3	87683.5	93897.6	59.27	0.0117
IP	377.7	25025.0	26237.8	54.83	0.0126
PO	912.0	64177.5	68526.0	59.10	0.0117

Pharmacokinetic profile of MS-0022 by IV, IP and PO administration in mice. The table displays the maximum concentration (Cmax), total drug exposure (AUClast) and (AUCinf), half-life (T1/2) and terminal slope (Kel).
doi:10.1371/journal.pone.0019904.t005

A possible solution to block Hh/GLI1 signalling despite activation mutations in the Smo locus would be to develop drugs that act on the pathway downstream of SMO [47]. A GLI inhibitor would also address non-canonical activation of GLI [57].

The high level of tumor-stroma interactions found in advanced cancers like pancreatic adenocarcinoma poses additional challenges for developing treatment strategies based on Hh antagonists. Such strategies would require that, in addition to autocrine efficacy, paracrine interactions between tumor activated stroma and tumor cells are disrupted [33]. Furthermore, the barrier that the stromal cells create around the tumor, and the lack of compound delivery to areas of low vascularization need to be addressed [41].

The physicochemical properties of MS-0022 (MW = 406.3; clogP: 5.44; PSA: 46.4 (Table 2)), its excellent per oral bioavailability, and the initial evidence that it is well tolerated *in vivo* (MTD >200mg/kg) makes MS-0022 a potential drug candidate. In PANC-1 and SUT-2 xenograft experiments, MS-0022 treatment led to a partial response, where growth was halted during the first days of treatment compared to the control. Over time, however, both the treated and control xenograft groups reverted to similar growth. In both SUT-2 and PANC-1 xenografts, the growth delay in the MS-0022 treated group resulted in a statistically relevant (P<0.05) average reduction in tumor volume of subsequently 27% and 38%. Our *in vivo* results lend support to the idea that the primary effect of Hh inhibition in pancreatic cancer treatment is due to the inhibition of Hh pathway activity in the stromal cells, although we also observe a mild initial reduction of GLI1 levels in tumor cells. Both pancreatic cancer cell lines, SUT-2 and PANC-1, that were xenografted as a part of this study, express high levels of Shh mRNA (Table 4), and should be able to induce a Hh response in stromal cells. It's interesting to note that though both MS-0022 and GDC-0449, and to a certain degree cyclopamine, led to a reduction of GLI1 mRNA in SUT-2 cells *in vitro*, suggesting the possibility to affect autocrine Hh signaling in this cancer cell line. In an *in vivo* context, the effect of MS-0022 *in vivo* on SUT-2 and PANC-1 tumors was most likely due to an inhibition paracrine Hh signaling since no statistically relevant reduction of either hGLI1 or hPTCH could be measured in mRNA derived from the tumor tissue. In contrast, mGli1 was reduced in the stroma of the SUT-2 tumors (P<0.05) after 7 days of treatment, but not at the end of the treatment, while at the end of the treatment in PANC-1 xenografts, there was a slight reduction of mGli1 (although with a P>0.05). Reasons for the lack of a measurable reduction of hGLI1 or hPTCH in the tumors, at the time points that were taken, could be low levels of compound reaching the tumor, or activation mutations that occurred within the tumors. Activation mutations in SMO have been observed in a recent clinical trial using CDG-0449 in a medulloblastoma patient [34,58]. Also, the development of resistance against chemotherapeutic agents in pancreatic cancer has been linked to both dense stromal matrix and increased stromal barrier, which over time, may cause resistance. Interestingly, Olive et al. found that treatment with IPI-926 led to an increased vascularization in KPC tumors, but similarly to our observations, the effect of this SMO inhibitor was due to a developing resistance [41].

KPC mice develop pancreatic tumors that resemble human pancreatic ductal adenocarcinoma due to a conditional expression of endogenous mutant Kras and p53 alleles in pancreatic cells [59,60]. Similarly, the SUT-2 and PANC-1 cell lines are mutant for p53 and Kras [61–65]. Despite the differences in the two models, with tumors forming in the mouse pancreas in the KPC model and tumor cells injected under the skin in the *in vivo* xenograft model,

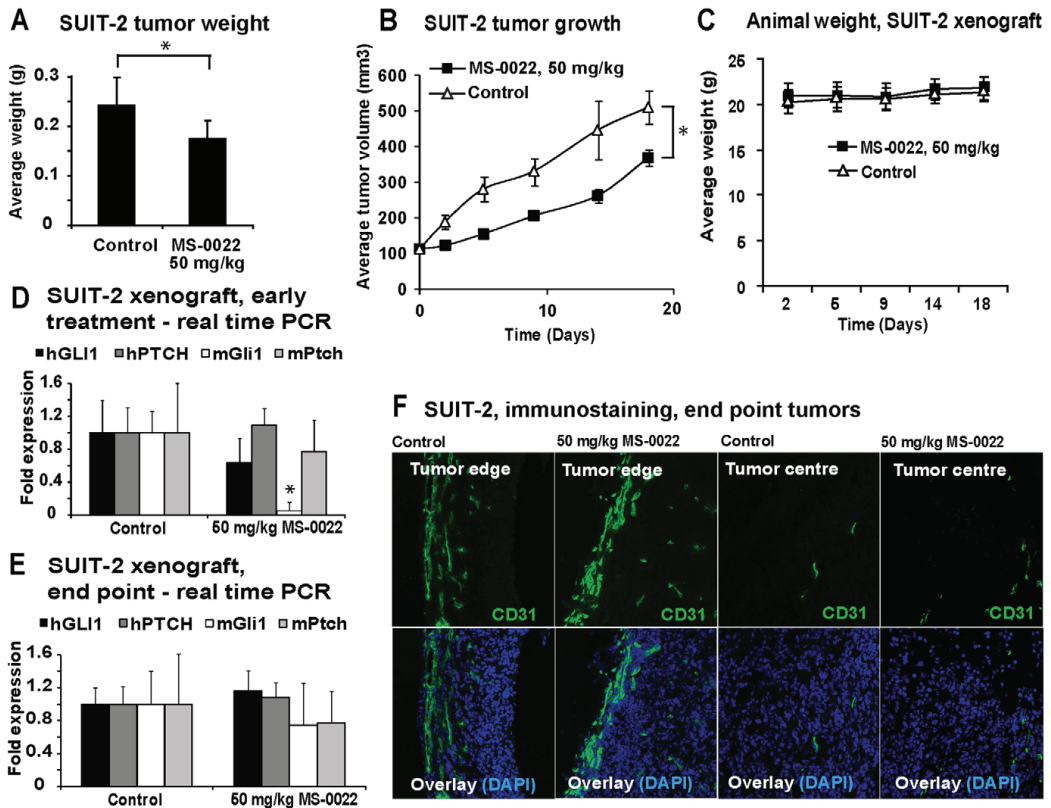


Figure 4. In vivo activity of MS-0022 in a SUIT-2 xenograft. A-F) Analysis of subcutaneous SUIT-2 tumors in mice treated with solvent control or 50 mg/kg MS-0022 for 7 (n = 3) or 18 days (n = 8) by PO administration. A) Graph displaying average SUIT-2 tumor weight at end of treatment, with SD. B) Tumor growth curve of SUIT-2 tumors in mice after 18 days of treatment, plotted as tumor volume (mm^3) with standard error of the mean (SEM). C) Average animal weight through the course of the treatment with SD (n = 8). D) and E) Graphs showing normalized (GUSB) fold expression of mouse and human Gli1 and Ptch in treated tumors with SD. D) Shows expression in tumors harvested after 7 days (n = 3), while E) shows expression in tumors harvested after 18 days (n = 8). F) Immunostaining of microvessels using anti-CD31 (endothelial cell marker) in cryosectioned tumor tissue slides. Images depict CD31 staining in the tumor edge and in the centre of the tumors. * = P value <0.05. doi:10.1371/journal.pone.0019904.g004

both studies provide evidence that the pancreatic cancer may only transiently be inhibited by a SMO antagonist, revealing that the challenge of overcoming resistance is ever present.

Materials and Methods

Ethics statement

All animals were housed and treated under the approved protocols in accordance with the National Institute of Health guide for the care and use of laboratory animals and according to regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2 and 3) and the conditions specified in *The Guide for Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996). Animal experiments performed in Norway were approved by the Centre for Comparative Medicine at the National Hospital of Norway according to the Regional Ethical Comity guidelines (permit nr: 19/08-910) and at the Norwegian Institute of Public Health (permit nr 2722), while animal experiments performed by

Chemdiv were approved by ChemDiv's Institutional Animal Care and Use Committee (Permit nr: 8/21.01.2008). All efforts were made to minimize the number and suffering of animals.

Cells and culture conditions

PANC-1, PC-3, THLE-2, NIH3T3, Shh-Light 2 (Shh-L2) and C3H10T1/2 cells were obtained from ATCC. SUIT-2 cells were obtained from Cell Bank, RIKEN BioResource Center. PTCH^{-/-} MEF [45] was a gift from P. A. Beachy and SUFU^{-/-} MEF were a gift from R. Toftgård (Karolinska Institutet). FEMX was a gift from G. E. Melandsmo. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ in the medium formulations as instructed by the suppliers.

Alkaline phosphatase assay and screening protocol

C3H/10T1/2 cells were seeded 5×10^3 cells per well in a 96-well format and allowed to attach for 3–5 hours, before adding serial dilutions of compounds (from a DMSO stock). After 30 minutes

recombinant human SHH (C24II) (RnD systems (1845SH)) was added (300 ng/mL). After 72 hours, the cells were lysed for 15 minutes using 20 μ L of a 10 mM Ethanolamine Buffer (pH 8.0) with 0.2% Triton-X100, supplemented 1:100 with Protease Inhibitor Cocktail (EMD Bioscience). 7.5 μ L of lysate was mixed with 45 μ L of CSPD Alkaline Phosphatase Substrate with Emerald-II (Applied Biosystems) and incubated at room temperature, in the dark, for 45 minutes. The plate was measured using a Wallac VictorV reader (0.1 s Luminescence). Screening compounds and MS-0022 were part of a Chemdiv chemical library.

Hh reporter assay

Shh-L2 cells were used as a Hh reporter cell line as described previously [45]. Hh signaling was induced by Shh conditioned medium, 200 nM SAG (AH-Diagnostics) and ActOXS (a combination of 5 μ M Hydroxycholesterol 20- α and 5 μ M Hydroxycholesterol 22S (Sigma-Aldrich)). For Shh conditioned medium, Shh-PANC-1 cells were grown to confluence and switched to fresh medium. Shh conditioned medium was harvested after 48 hours, and was diluted to 50% in fresh DMEM before use. The clonal Shh-PANC-1 cell line was generated by stably transducing PANC-1 cells with virus containing medium from HEK293T cells that were transfected using a Virapower Lentiviral expression kit (Invitrogen). The vector was produced by removing GFP from a pLenti 6.2-vector, and inserting the mouse Shh cDNA sequence.

IC50 calculation

IC50 values were calculated using dose response curve data where $n=3$. The calculations were performed with an online calculator using the formula: $a \exp(-bx) + c$ at the website; <http://www.changbioscience.com/stat/ec50.html>

NMR spectroscopy of MS-0022

NMR spectra of MS-0022 (approx. 2 mg) were obtained from a solution in 0.5 ml of DMSO- d_6 ((CD₃)₂S(O), 99.9% D, Cambridge Isotope Laboratories, Andover, MA) in a 5.0 mm tube (WILMAD, WG-5 Economy). The spectra were acquired on an Avance AV 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) with a 5 mm CP-TCI (1H/13C, 15N-2H) triple-resonance inverse cryo probe, equipped with a Z-gradient coil. NMR assignments of MS-0022 were inferred from examination of ¹H- and ¹³C spectra, attached proton test (APT), correlated spectroscopy (COSY45), total correlation spectroscopy (TOCSY) pulsed field gradient heteronuclear single quantum coherence (g-HSQC), pulsed field gradient heteronuclear multiple bond correlation (g-HMBC) and pulsed field gradient heteronuclear two-bond correlation (g-H2BC/g-HSQC-COSY). The data were processed using the Bruker TOPSPIN software (version 1.3 or version 2.1 pl 2). Chemical shift values were referenced to the residual solvent signals, i.e. CHD₂S(O)/CD₃=2.49 ppm and (CD₃)₂S(O)=39.5 ppm, respectively. All NMR spectra were acquired at 25°C.

BODIPY assay

BODIPY assay was performed to determine competition with cyclopamine as described previously [21,66].

Real Time PCR

Cells were lysed for RNA extraction after 48 or 72 hours of treatment. Total RNA from cultured cells or tumor tissue was isolated using the GeneElute miniprep kit (Sigma-Aldrich) following the manufacturer's instructions. cDNA was synthesized

from the isolated mRNA using the Retroscript kit (Ambion), and real-time PCR was carried out using the SYBR Green PCR master mix (Stratagene) according to the manufacturer's instructions with an Mx3000P cyclor (Stratagene). The relative concentrations of cDNA present in each sample were calculated by the MxPro software (Stratagene), normalized for GAPDH (mRNA from cells) or GUS (mRNA from tumors). For real time PCR primer sequences see Table 6. For the tumor tissue real time PCR, previously described human and mouse specific primers against GUSB, PTCH1 and GLI1 were used [33].

Immunofluorescence

PTCH^{-/-} MEF were seeded on pre-coated glass slides (1 hour coating with 0.1% Gelatin (G1393) and 0.003% Collagen (C8919) (Sigma-Aldrich) in PBS). 80% confluent cells were switched from 10% to 0.5% FBS in medium and cells were treated with DMSO or 5 μ M MS-0022 for 24 hours ($n=3$) before subsequent immunofluorescent staining. Slides were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized for 10 min in 0.1% v/v Triton X-100 (Sigma-Aldrich) in PBS (PBT), and blocked with 10% w/v BSA (Savcen Werner) in PBT for 1 hour. Primary antibodies, mouse anti-acetylated tubulin (α -Tub) (Sigma-Aldrich (T7451), 1:1000) goat anti-Smo (Santa Cruz Biotechnology (sc-6367), 1:1000), and goat anti-GLI-1 (RnD systems (AF3455), 1:500) were diluted in 0.5% w/v BSA in PBT and left on the slides over night at 4°C. Secondary antibodies donkey anti-goat IgG-Alexa 594 and donkey anti-mouse IgG-Alexa 488 (Invitrogen, both at 1:500) were added in 0.5% w/v BSA in PBT for 1 hour. The slides were counterstained with DAPI (Roche,

Table 6. Primer sequences for quantitative real time PCR.

Primers:	Primer sequence, 5' \rightarrow 3'	Tm, °C
hPTCH1 fwd	CGA GCC CCC CTG TAC GAA GTG G	67.7
hPTCH1 rev	GAC CCC CAG CAA GCC CAG AAA A	64.0
hGLI1 fwd	GCG CAT CCC GAG CCC AGC	65.1
hGLI1 rev	GCC CTC GGT GCA GCT GTT GGT C	67.7
hGLI2 fwd	GCC TCC GAG AAG CAA GAA GCC AAA A	64.4
hGLI2 rev	CCT GGT GTC GCA TGT CAA TCG GTA G	66.3
hGLI3 fwd	CGG GAC GGT GTT TGC CAT GGA C	65.8
hGLI3 rev	GGA GGA TGG AAG GCA GCG AAA AGA T	64.6
hSHH fwd	GCC AGC GGA AGG TAT GAA GGG AAG	66.1
hSHH rev	ACC GAG ATG GCC AAA GCG TTC AAC	64.4
hHIP fwd	TGG GGA TGG CTC GCA ACG TCT C	65.8
hHIP rev	TGG GAT GGA ATG CGA GGC TTA GC	64.2
hSUFU fwd	CCC GAG GAT GAG GAG CAG AGC C	67.7
hSUFU rev	CGC GTG CGA ATC AGC TCA TGG G	65.8
hSMO fwd	CCA GGA GGA AGC GCA CGG CAA G	67.7
hSMO rev	TCG CAC TGG CCT GAA CTG TTG AAC T	64.6
hGAPDH fwd	GCC CCC TCT GCT GAT GCC CCC A	75.7
hGAPDH rev	TGG GTG GCA GTG ATG GCA TGG	70.2
mGLI1 fwd	AGC CAA CTT TAT GTC AGG GTC CCA GGG T	71.2
mGLI1 rev	GAG CCC GCT TCT TTG TTA ATT TGA CTG	66.5
mGAPDH fwd	TAT GTC GTG GAG TCT ACT GGT GTC TTC ACC	68.1
mGAPDH rev	GAG TTG TCA TAT TTC TCG TTG TTC ACA CCC	66.8

Table of real time PCR primers; name, sequence and Tm°C (h = human, m = mouse).

doi:10.1371/journal.pone.0019904.t006

1 mg/ml) in PBS. 3 random regions were imaged per slide using a confocal LSM510 microscope (Carl Zeiss MicroImaging). The ratio of green Smo positive cilia versus the red aTub positive cilia was quantified manually or processed and analyzed using ImageJ to quantify the intensity of Smo and aTub for each cilium in the images (<http://rsb.info.nih.gov/ij/>). Graphs display mean with standard deviation error bars.

Frozen tumor tissue was cryosectioned ($n = 8$ pr group), and the sections were fixed with 4% paraformaldehyde for 10 minutes before permeabilization with 0.2% Triton-X in PBS for 10 minutes. Sections were blocked for 30 minutes at room temperature with 3% BSA in PBS and was subsequently incubated over night at 4°C with rat anti-mouse CD31 (BD Biosciences (550274), 1:500) in 1% BSA in PBS. The secondary antibody used was Alexa Fluor 488 goat-anti-rat (Molecular Probes; 1/700 dilution) in 1% BSA in PBS for 1 hour at RT. Nuclei were counterstained with DAPI, in PBS for 10 minutes at RT. Images was acquired as above and electronic images was further processed using ImageJ.

Transfection and luciferase assay

Vectors were obtained from the following: GliBS-Luc reporter [67] (gift from H. Sasaki), pBluescript (pBS) (Stratagene), FL-HA-GLI1 (Gli1) and Gli2-GFP (Gli2) (gifts from R. Toftgård), Super8XTOPFlash [68], and NFκB-luc (Panomics). 10 ng renilla luciferase (pRL-TK) (Promega) was used as a transfection control. HEK293 and NIH3T3 cells were seeded at 80,000 per well in 48-well plates on day 1, and were co-transfected on day 2 using a total of 0.4 μg plasmid per well mixed at 1:3 in FUGENE6 according to manufacturer's instructions. On day 3, control medium and activating medium (50% L1 conditioned medium, 50% Shh conditioned medium or medium containing 10 ng/mL recombinant $\text{rrTNF-}\alpha$ (R&D Systems)) \pm 10–20 μM MS-0022 were added, using DMSO as a control (the NFκB pathway inhibitor Bay11-7082 was used as a control in the NFκB assay). Samples were analyzed on day 4 or 5 using the Dual Luciferase assay kit according to manufacturer's instructions. Graphs display relative or fold reporter activity, and are calculated from a mean ratio of firefly reporter luminescence/pRL-TK luminescence with standard deviation error bars ($n = 3$). The experiments have been repeated with similar results.

In vitro antiproliferative assay

Cells were plated at a density of 3000 (THLE-2 and PC-3) and 1000 (PANC-1, SUIT-2 and FEMX) cells per well (on the basis of their growth rate) in 200 μl of medium in a 96-well plate. Medium was changed daily, and after four days, the assay was read using a MTS kit (Promega) according to the instructions of the manufacturer. Graphs display average of percent growth with standard deviation ($n = 8$).

Passage growth assay

20,000 cells were seeded per well in 6 well plates (triplicates) with DMSO or 5 μM MS-0022. Treatment medium was changed on day 3–5, and after 5–7 days the cells were trypsinized, resuspended in medium and counted, before reseeding cells at 20,000/well for a subsequent passage. The average growth of MS-0022 treated cells was calculated as a percentage of the growth of the DMSO control at the end of each passage. Graphs display average of percent growth with standard deviation bars ($n = 3$).

Soft agar colony formation assay

2000 PANC-1 or SUIT-2 cells were suspended in 1.5 mL growth media containing 0.35% agar (Oxoid), over a 1.5 mL

base layer containing 0.5% agar in 6-well plates. The plates were incubated for 14 days (SUIT-2) and 21 days (PANC-1) and then stained for 1 h with 1 ml of 0.02% crystal violet (Sigma-Aldrich), and colonies were counted according to sizes; <0.2 mm (small), 0.2–1 mm (medium) and >1 mm (large). Data represent average numbers with standard deviation bars ($n = 3$). The experiments have been repeated with similar result.

Pharmacokinetics, dose tolerance, animal tumor establishment and treatment

For pharmacokinetic (PK) analysis, 5 mg/kg MS-0022 was given by IV, PO and IP administration to 7–8 week old male C57BL mice (*mouse musculus*), and blood was collected after 5, 15, 30, 60, 120, and 240 min, with $n = 3$. Animals were sacrificed and blood samples (~0.5 mL each) were collected from the abdominal aorta into EDTA-containing tubes, centrifuged (2–8°C for ~10 minutes at ~10000 $\times g$) and plasma harvested into single tubes for each animal and frozen (~−70°C). Blood samples were analyzed by ChemDiv's bioanalytical department. The maximum concentration (C_{max}), total drug exposure (the area under the curve to the last quantifiable concentration (AUClast) and as the AUClast value extrapolated to infinity (AUCinf), calculated as $\text{AUCinf} = \text{AUClast} + C(t)_{\text{last}}/K_{\text{el}}$, where $C(t)_{\text{last}}$ is the last measurable concentration, half-life ($T_{1/2}$) calculated as $\ln(2)/K_{\text{el}}$ and K_{el} calculated as the slope of the terminal linear portion of the concentration/time curve. The WinNonlin Professional 5.2 software (Pharsight Corp.) was used for the calculation of the PK parameters.

For the analysis of uptake of MS-0022 in organs and tissue, livers were dissected out from mice 120 min after PO administration of MS-0022 and frozen (~−70°C). The LC-MS method used to detect MS-0022 was described previously [69], with the main exception being that isocratic conditions were used (50% 0.1% FA (aq), 50% 0.1% FA (ACN), v/v), and that a UV detector was placed between the LC and MS instrument.

MS-0022 tolerance was tested by daily injections of 200 mg/kg MS-0022 for 5 days in 7–8 week old male C57BL mice. Animal were observed daily for outward signs of toxicity (weight loss, fur ruffling, hunched posture).

For PANC-1 xenografts, 4–6 week old CB17/SCID mice were injected with 5×10^6 PANC-1 ($n = 6$ per group) cells in 200 μl PBS subcutaneously on day 1. IP treatment with 50 mg/kg MS-0022 began when median tumor size reached 25 mm² (day 27). MS-0022 was pulverized using a pestle, and was mixed into a stable suspension in water containing 1% Tween 80 (Sigma-Aldrich). Mice were treated with MS-0022 or solvent for a period of 30 days after initiation in a 5 day injection 2 day pause scheme, and tumor size was measured throughout using calipers (mm²). After 30 days the mice were sacrificed and tumors dissected and weighed. The PANC-1 xenograft endpoint was taken two days after the last compound treatment.

For SUIT-2 xenografts, 4–6 week old CB17/SCID mice were injected with 5×10^6 SUIT-2 ($n = 11$ per group) cells in 200 μl PBS subcutaneously on day 1. IP treatment with 50 mg/kg MS-0022 began when median tumor size reached 35 mm² (day 8). Mice were treated daily with MS-0022 or solvent for a period of 18 days after initiation, and tumor size was measured throughout using calipers (mm²). After 8 days, three mice from each group were sacrificed and tumors dissected, weighed and processed for analysis. The remaining animals ($n = 8$) were sacrificed after 19 days of treatment, and tumors harvested. Tumor volume (mm³) was calculated using the formula $a \times b^2 \times \pi/6$. End tumor measurement statistics were obtained using two samples T-test in the Sigmaplot software.

Liver transaminases

Blood was collected from the hepatic vein of freshly sacrificed animals into heparinized capillary blood collection tubes (Sarstedt), which were immediately centrifuged and the serum was collected. AST and ALT were run on non-hemolyzed samples using AST and ALT kits (Randox) following the manufacturer's instructions.

Supporting Information

Figure S1 Atom numbering of MS-0022. The atom numbering of MS-0022 used in the ^1H and ^{13}C NMR analysis. (See Table S1) (TIF)

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Table S1 NMR analysis of MS-0022. ^1H and ^{13}C NMR Data for MS-0022 (DMSO- d_6). For the atom numbering used see Figure S1. (DOC)

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Author Contributions

Conceived and designed the experiments: MFS SRW JLD DDH AK IO DP SK. Performed the experiments: MFS SRW JLD AK IO DP. Analyzed the data: MFS SRW JLD DDH AK IO DP SK. Contributed reagents/ materials/analysis tools: MFS SRW JLD DDH AK IO DP. Wrote the paper: MFS SRW JLD DDH AK IO DP SK.

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